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Determination of Neural Crest Cell Fate at Neural Tube vs. Target Organ Level

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TABLE OF CONTENTS

ABSTRACT

The neural crest is a transient pool of multipotent progenitor cells that give rise to different cell types within the vertebrate embryo. Trunk neural crest cells emigrate from the dorsal neural tube. They differentiate into the sensory and autonomic neurons, Schwann cells, chromaffin cells of adrenal medulla, and melanocytes. The neural crest derived sympathetic neurons, chromaffin cells, and small intensely fluorescent cells have been proposed to share a common sympathoadrenal progenitor. NGF has been reported to be important for the differentiation of the neuronal lineage and glucocorticoids for the differentiation of the endocrine lineage. However, based on analyses of mice lacking the glucocorticoid receptor or the adrenal cortex, glucocorticoids have been found to be necessary for the survival of adrenal chromaffin cells but not for their differentiation. *In situ* hybridisation analysis of different neuronal and adrenal markers has revealed heterogeneity of sympathoadrenal cells before invading the adrenal medulla and sympathetic ganglia, respectively. These findings challenged the classical hypothesis of chromaffin cell differentiation and suggested that adrenomedullary cells and sympathetic neurons might have distinct progenitors. We attempted to elucidate whether the fate of the chromaffin cells and the sympathetic neurons is predetermined at the level of the neural tube. To prove our hypothesis, we performed a single neural crest cell electroporation technique in combination with immunohistological analysis. Our results from single cell electroporation experiments strongly supported the hypothesis that chromaffin cells and sympathetic neurons originate from a single progenitor cell at the level of the neural tube. However, we cannot exclude that few sympathetic neurons and chromaffin cells have distinct progenitors. In the second part of this study, we aimed to screen for expression patterns for Notch signalling members. Based on the fact that Notch signalling is involved in sympathetic ganglia development, we attempted to investigate the potential involvement of Notch signalling in the differentiation of sympathoadrenal precursors toward neuronal and neuroendocrine derivatives. In the last part of the thesis, we have investigated the differences in the vascular pattern of adrenal gland and sympathetic ganglia in developing avian embryos. The environmental signals of adrenal gland and sympathetic ganglia might provide factors that influence the sympathoadrenal differentiation.

Keywords: neural crest, sympathoadrenal lineage, chromaffin cell, sympathetic neuron, electroporation, chicken

ZUSAMMENFASSUNG

Die Neuralleiste ist eine embryonale Struktur von multipotenten Vorläuferzellen, aus denen verschiedene Zelltypen entstehen. Die Neuralleistenzellen wandern aus dem dorsalen Neuralrohr und differenzieren sich u.a. in sensorische und autonome Neurone, Schwann-Zellen, chromaffine Zellen der Nebennieren und Melanozyten. Neuroendokrine chromaffine Zellen, sympathetische Neurone und SIF Zellen entstehen nach traditioneller Vorstellung aus einer gemeinsamen sympathoadrenalen Vorläuferzelle. Spekuliert wurde, dass NGF eine wichtige Rolle im Differenzierungsprozess der neuronale Zelllinie spielt, während Glucocorticoide das neuronale Differenzierungsprogramm unterdrücken und chromaffine Zellen entstehen lassen sollen. Die Analyse von GR-/- Mäusen hat die Rolle von Glucocorticoiden in dem Differenzierungsprozess von chromaffinen Zellen nicht bestätigt. In situ Hybridisierungs-Analysen von neuronalen und neuroendokrinen Markern suggerierten eine Heterogenität von sympathoadrenalen Zellen vor der Einwanderung in ihre Zielorgane. Daraus entwickelten wir die Hypothese, dass es möglicherweise verschiedene Vorläuferzellen von sympathetischen Neuronen und chromaffinen Zellen gibt. Wir wollen in dieser Arbeit klären, ab die beiden Zelltypen von einer oder zwei verschiedenen Vorläuferzellen abstammen. Wir elektroporierten einzelne Neuralleistenzellen kurz vor der Delaminierung und analysierten die Tochterzellen immunohistochemisch mit spezifischen Markern. Unsere Ergebnisse haben die klassische Hypothese einer gemeinsamen sympathoadrenalen Zelllinie stark unterstützt. Wir können aber nicht ausschliessen, dass einige sympathetische Neurone oder chromaffine Zellen von zwei verschiedenen Vorläuferzellen abstammen. Wir haben begonnen zu untersuchen, wie distinkte Phänotypen von sympathischen Neuronen und chromaffinen Zellen entstehen, indem wir die Expression von Molekülen des Notch-Komplexes und die frühen vaskulären Muster in den Anlagen von sympathischen Ganglien und Nebennieren analysiert haben.

Schlüsselbegriffe: Neuralleiste, sympathoadrenale Zelllinie, chromaffine Zellen, sympathetische Neuronen, Elektroporation, Hühnchen

1 INTRODUCTION

1.1 Neural crest

The neural crest (NC) is a transient pool of progenitor cells that extends along the dorsal neural tube and gives rise to a large scale of different cell types within the vertebrate body (Le Douarin and Kalcheim, 1999). The neural crest represents an important model for studies of developmental processes as cell migration, proliferation, and differentiation (Le Douarin and Kalcheim, 1999). The NC cells undergo epithelial-to-mesenchymal transition, acquire the possibility to move, migrate along defined routes toward the target sites where they settle and differentiate (Burstyn-Cohen and Kalcheim, 2002). During neurulation, the ectoderm layer subdivides into the prospective epidermis (non-neural part) and the neural plate (neural part). At the border between the neuroectoderm and the non-neural ectoderm, the neural crest is formed. Numerous transcription factors have been implicated in its formation and induction (see Fig.1). Among them, Wnt, FGF and BMPs belong to the most intensely studied signals important for NC formation (Kalcheim and Burstyn-Cohen, 2005).

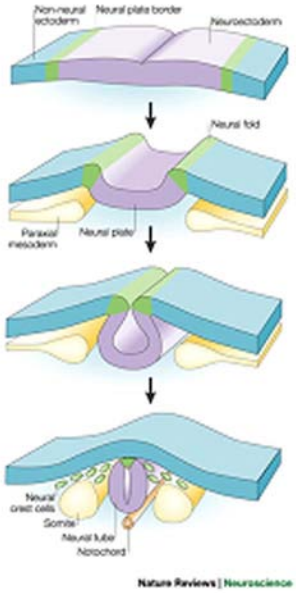


Fig 1.1: Neural crest induction. The neural crest (green) is induced at the border between the neuroectoderm (purple) and the non-neural ectoderm (blue). NC cells (green) delaminate from the neural tube (Gammill and Bronner-Fraser, 2003).

1.2 Fate of neural crest cell derivatives

1.2.1 Derivatives of NC

The neural crest progenitors produce both epithelial and mesenchymal derivatives such as autonomic, sensory, enteric neurons and glial cells, neuroendocrine cells of thyroid and adrenal glands, smooth muscle cells, cartilage, skeleton and connective tissue of the head, and all melanocytes except retinal pigment cells (Le Douarin and Kalcheim, 1999).

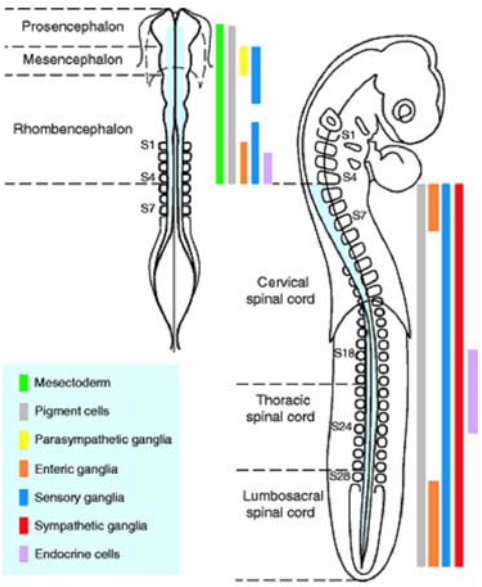


Fig. 1.2: Fate map of the neural crest-derived phenotypes along the neural axis in the chick model. The chicken embryo of seven somite pairs old on the left side depicts tissues that arise from the cephalic NC. The scheme of an embryo of 28 somite pairs on the right side shows tissues that arise from the trunk NC in cervical, thoracic and lumbosacral regions of the spinal cord (Le Douarin, Creuzet et al., 2004).

The neural crest fate map depicted in Fig. 1.2 shows the various cell phenotypes yielded by NC cells at different antero-posterior levels of the neural tube. Melanocytes are produced along the entire length of the neural axis. The parasympathetic ciliary ganglion (yellow) derives from the mesencephalic NC. Enteric ganglia arise from both vagal (somite 1-somite 7) and lumbosacral (posterior to somite 28) NC. The trunk NC located caudal to somite 4 yields sympathetic ganglia (red). The regions that give rise to the sensory ganglia (dark blue) are the mes-metencephalic NC and the NC from posterior rhombencephalic to lumbosacral levels. Thyroid C cells (violet), the

neuroendocrine cells that produce calcitonin, originate from the NC of somite 2 - 4. The chromaffin cells of adrenal medulla are generated from the NC of the level between somite pairs 18 and 24, so called “adrenomedullary” somite level (Le Douarin, 1999; Le Douarin, Creuzet et al., 2004). The trunk NC gives rise to sensory and autonomic neurons and glial cells (both ganglionic satellite and Schwann cells) of peripheral nervous system, chromaffin, SIF and pigment cells.

1.2.2 Interplay of intrinsic and extrinsic factors

The understanding of the specific mechanisms underlying the development of an organism from a single fertilized cell and subsequent cell fate specification during embryogenesis has attracted the interest of developmental biologists for decades. Till now, the general conception of importance of both environmental factors and cell autonomous programs in cell-fate specification is accepted. There are two models of NC cell differentiation perceived among the scientists. One group proposes that NC cells are multipotent and differentiate according to the environmental conditions (Bronner-Fraser, Stern et al., 1991; Fraser and Bronner-Fraser, 1991; Le Douarin and Dupin, 2003). The others suggest that NC cells become restricted in fate early in their existence, and they attribute a bigger role to cell-autonomous programs (Erickson and Goins, 1995; Harris and Erickson, 2007).

1.2.3 Neural crest as a heterogeneous cell population

The neural crest, as a whole, is described as a heterogeneous population of cells, composed of both multipotent and restricted precursors (Harris and Erickson, 2007). Studies on neural crest cells have aimed to elucidate to what extent NC cells are multipotent, what is the role of developmental gene combinatorial code and its relation to external signals. These experiments have revealed pluripotent capacity of early neural crest cells as well as a defined portion of some restriction in fate (Le Douarin and Kalcheim, 1999). It is important to make clear that restriction in fate is defined as a specification. This is different from restriction in potential, or commitment, which means the capacity to produce different cell types under the different environmental conditions. Some neural crest cells may be restricted before delamination but that does not necessarily mean they have lost the potential to form another cell type (Dorsky, Moon et al., 2000).

1.2.4 Temporal and spatial factors influencing fate of NC derivatives

The diversity of cell types within NC derivatives has been shown to depend on time and space. Spatial heterogeneity in cell fate among premigratory NC cells has been described in the rostro-caudal direction (Le Douarin, Creuzet et al., 2004). The quail-chick chimera system has brought a great contribution to the establishment of the NC fate map along the antero-posterior axis. This system was established and performed by Le Douarin who noticed that the interphase nuclei of quail cells contained a large amount of heterochromatin. This is not the case in chick cells as the heterochromatin is equally distributed within the nucleoplasm. This feature allows to distinguish easily between the quail and chick cells, respectively (Le Douarin and Kalcheim, 1999). As depicted in Figure 1, cell progenitors derived from different axial levels produce variable cell types and spread through define routs of migration (Le Douarin, Creuzet et al., 2004).

The importance of the time in the NC cell specification has been apparent from many studies (Serbedzija, Bronner-Fraser et al., 1994; Henion and Weston, 1997; Harris and Erickson, 2007). *In vitro* clonal analysis of trunk NC cells has revealed segregation of neurogenic and melanogenic sublineages in time. Melanogenic progenitors emigrated from the neural tube after neurogenic precursors. This study has further shown that many NC cells are fate restricted at the time of delamination from NT (Henion and Weston, 1997).

1.3 Migration of trunk neural crest cells

Several methods have been performed to study the timing and the pathways of NC cells including injections of vital dyes, immunolabelling of serial sections or *in vivo* labelling with fluorescent markers. The human natural killer lymphocyte antigen HNK is expressed by migrating neural crest cells. The HNK-1 (synonymous CD57) monoclonal antibody allows to immunolabel the NC cells after they detach from the neural tube (Bronner-Fraser, 1986; Serbedzija, Bronner-Fraser et al., 1989; Langley and Grant, 1999).

Studies of NC cells migration to the rat embryonic adrenal medulla have described the behaviour of migrating cells by three-dimensional reconstruction (Yamamoto, Yanai et al., 2004).

Kulesa and colleagues monitored the pattern of NC cell-migration by life cell time-lapse imaging. They have shown the importance of communication between migrating cells (Kasemeier-Kulesa, Bradley et al., 2006).

1.3.1 Delamination of neural crest cells

Migration of NC cells depends on a coordinated action of cytoskeletal and cell adhesion molecules, extracellular matrix components, and transcription factors. The emigration of NC cells from the dorsal midline of the neural tube and their migration is a well-controlled process. Regulation of cell cycle and balance between BMP and its inhibitor noggin have been shown to play an essential role (Le Douarin and Kalcheim, 1999).

In chicken, the delamination of the trunk neural crest cell from the dorsal midline in the neural tube starts opposite to the epithelial somite. There is no delamination opposite to the segmental plate. The mechanism that controls the timing of NC cells delamination along the rostro-caudal axis involves BMP signalling and is explained by the interaction between neuroepithelium and paraxial mesoderm. The somite development is actively connected with the onset of NC cells emigration from the neuroepithelium. The delamination is triggered by BMP4 activity and negatively regulated by noggin (Sela-Donenfeld and Kalcheim, 2000). Noggin is expressed in the dorsal neural tube and forms a gradient along the rostro-caudal axis. The highest expression of noggin could be detected caudally opposite to the unsegmented mesoderm, where the neural crest cells are still non-migratory, and decreases rostrally opposite to the epithelial somites and somites that start to dissociate into sclerotome and dermomyotome (Sela-Donenfeld and Kalcheim, 1999). The transcription of *noggin* gene is inhibited by an unidentified factor that is produced in the dorso-medial region of the developing somite. As somites differentiate, noggin is inhibited, BMP4 is active and NC cells detach from the neural tube (Sela-Donenfeld and Kalcheim, 2000). In avian embryos, the cessation of delamination of trunk NC cells is finished within in approximately 48 hours after the onset of migration (Erickson, Duong et al., 1992; Kalcheim and Burstyn-Cohen, 2005).

The NC cells proliferate when residing in the dorsal neural tube, and they are also mitotically active during migration. *In vitro* studies of cell cycle changes during trunk NC cell differentiation have explored the cell cycle parameters. The average cell cycle parameters for chicken NC cultured cells were described as follows: S-phase=4,4 hours, G1=3,8 hours, G2=1,5 hours, and M=1,7 hours (Maxwell, 1976). Cells in the

neuroepithelium undergo interkinetic nuclear migration meaning that the position of the cell nucleus changes according to the phase of the cell cycle. The cell cycle duration of neuroepithelial cells lasts about eight hours in avians (Kalcheim and Burstyn-Cohen, 2005). The trunk NC cells emigrate from the dorsal midline synchronously in the S phase of the cell cycle. The successful transition from phase G1 to the phase S was shown as necessary for the onset of NC cells migration. This was proven by inhibition of the G1-S transition, which prevents the delamination of NC cells. The premigratory NC cells become mesenchymal by the process of epithelial-to-mesenchymal transition (EMT) and acquire the capability to move. The transition from G1 to S phases is an indispensable step for EMT and thereby for the delamination of NC cells from the neuroepithelium (Kalcheim, 2000; Burstyn-Cohen and Kalcheim, 2002).

1.3.2 Ventral pathway

The neural crest cells migrate along two main pathways in the trunk of vertebrate embryos. These two dominant routes have been named according to the direction in which they the cells move: the ventral and the dorso-lateral pathway. First, the undifferentiated NC cells enter the ventral pathway, migrate along defined pathways and occupy the positions of sympathetic and sensory ganglia, adrenal medulla, and enteric nervous system (Le Douarin and Kalcheim, 1999). The HNK1-positive cells have been shown to migrate ventrally through the defined routes, ie. through three different pathways: 1) between individual somites, 2) through the anterior part of a somite, and 3) between the neural tube and the posterior sclerotome (Bronner-Fraser, 1986; Kalcheim, 2000). The active locomotion of NC cells through the ventral pathway has been suggested (Loring and Erickson, 1987). The NC cells are guided through the somite by neuropilin-semaphorin signalling. The migrating cells enter only the anterior half of the somite as they express the neuropilin-2 receptor; its repulsive ligand semaphorin-3F is restricted to the posterior half of somite (Gammill, Gonzalez et al., 2006).

All ventrally migrating NC cells also express, in addition to neuropilin-2 receptor, also ephrin-B3 (EphB3) receptor. EphB3 is a receptor tyrosine kinase that binds to its ligand ephrinB1. EphrinB1 is localized to the posterior half of the somite and, similar to semaphorin-3F, provides repulsive signals that block invasion of the posterior part of somite by NC cells (Harris and Erickson, 2007).

Several other molecules are involved in anterior-posterior segmental pattern of NC cells migration. The extracellular matrix protein F-spondin and PNA-binding glycoproteins are expressed in the posterior half of somites. They interact with ventrally

migrating crest cells and instruct them to the anterior segment of somite. The analysis of mice mutated for neuropilin-2 or semaphoring-3F showed disrupted segmental pattern of neural crest cells migration (Gammill, Gonzalez et al., 2006).

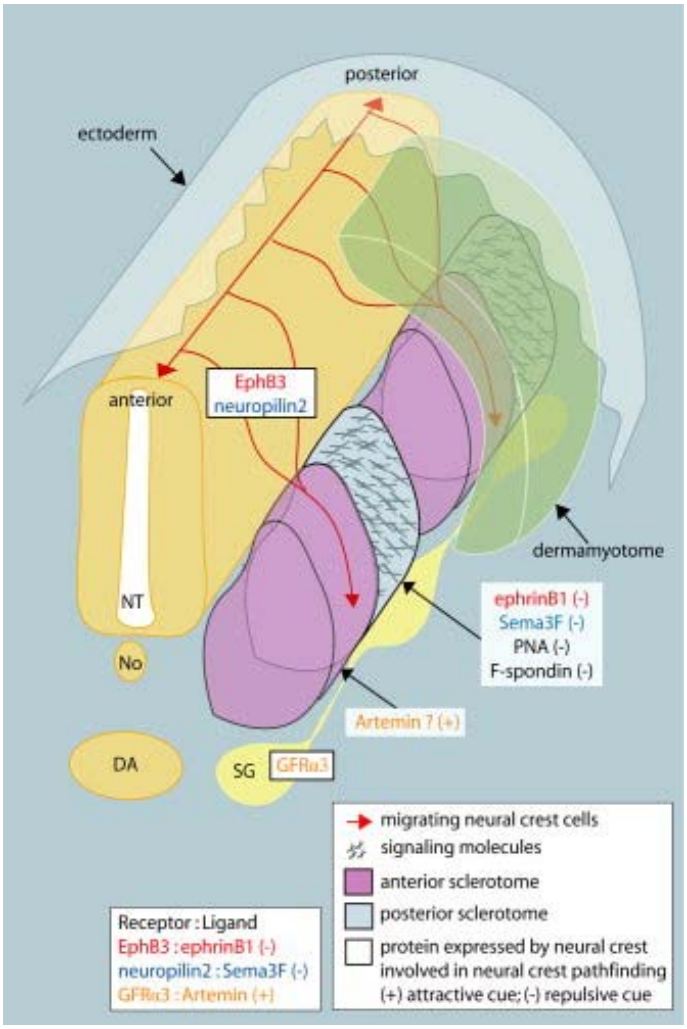


Fig 1.3: Ventral pathway and proteins involved in anterior-posterior segmentation (Harris and Erickson, 2007).

1.3.3 Dorso-lateral pathway (subectodermal)

NC cells that detach from the neural tube and enter the dorso-lateral pathway do so after the ventral pathway is already occupied. During chick normal development, the dorso-lateral path is open about 24 hours after the onset of NC cells emigration (Erickson, Duong et al., 1992). The delay in the dorso-lateral dispersal of NC cells is explained by the importance of cell-matrix adhesion molecules, which are expressed on melanocytes, chemotactic factors or extracellular matrix components. The pigment cells, as the only NC derivatives, travel along the second pathway. It has been shown

that the NC cells specified as melanocytes are able to enter the subectodermal path, whereas the other NC derivatives are excluded. This study has shown that pigment cell precursors are sublineage of neural crest that is directed to a specific pathway on the basis of their phenotype (Erickson and Goins, 1995).

1.4 Sympathoadrenal cell lineage

1.4.1 Sympathetic neurons, chromaffin and SIF cells

It is widely assumed that the neural crest derived sympathoadrenal (SA) cell lineage is a common source for adrenergic cell types including sympathetic neurons, small intensely fluorescent (SIF) cells, and chromaffin cells of both adrenal medulla and extra-adrenal paraganglia (Anderson, 1993). The largest and well documented extra-adrenal chromaffin cell accumulations described are represented by the organ of Zuckerkandel and carotid body (Unsicker, Huber et al., 2005). All cells committed to the SA lineage share several features. They all are able to synthesize and store catecholamines. They are characterized by expression of tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine biosynthesis. In contrast to these similarities, derivatives of SA lineage also possess features specific to every individual cell type. The similarities and differences among the SA derivatives are schematically summarized in Figure 1.4 (Doupe, Landis et al., 1985).

Sympathetic neurons

The neurons of the sympathetic autonomic nervous system are mainly located in sympathetic ganglia, although some of them can be found in adrenal glands (Langley and Grant, 1999). The most characteristic morphological features of sympathetic neurons are axons and dendrites. Sympathetic neurons can be specifically distinguished from other types of neurons by markers of catecholamine synthesis; the most widely used is the enzyme tyrosine hydroxylase (Le Douarin, 1999).

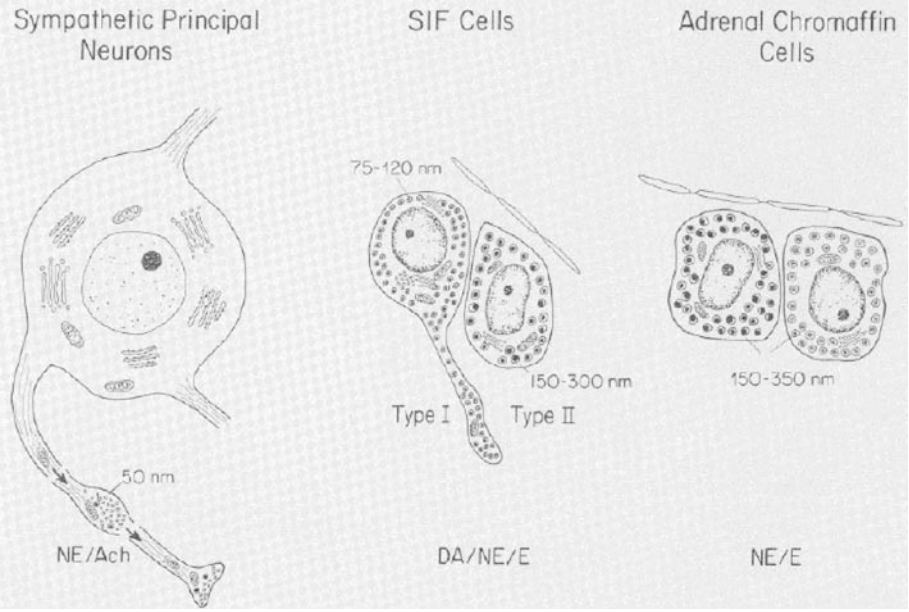


Fig 1.4: Scheme of the three adrenergic derivatives of the neural crest, their morphological similarities and differences (Doupe, Patterson et al., 1985).

Chromaffin cells

Neuroendocrine cells of adrenal medulla are called chromaffin cells according to their affinity to chromium salts (Unsicker, Huber et al., 2005). Differentiated chromaffin cells differ from sympathetic neurons, as they do not express the pan-neuronal marker neurofilament. Adrenomedullary cells are smaller in diameter than sympathetic neurons and they are characterized by the presence of large dense-core vesicles (Unsicker, Habura-Fluh et al., 1978). These large catecholamine-storing vesicles are responsible for intense fluorescence induced by the reaction between formaldehyde and amines (Eranko, 1955). Based on the cytofluorescent technique two chromaffin cell types have been distinguished, one giving stronger fluorescence signals than the other and reflecting noradrenaline (NA) and adrenalin (A) storing cells, respectively. NA-containing cells fluoresce more strongly. The A-containing chromaffin cells contain additionally phenylethanol-amine N-methyltransferase (PNMT), the enzyme necessary for the synthesis of adrenaline (Langley and Grant, 1999). Recently, the presence of chromaffin-like cells in sympathetic ganglia of chicken has been described. The chromaffin cells comprise the proportional part of about 25% of all TH-positive ganglion cells in avians (Huber, Franke et al., 2008).

Small intensely fluorescent cells

SIF cells, also termed as small granule containing cells (SGC), represent a cell type with intermediate phenotype between sympathetic neurons and chromaffin cells. They extend neurites (type I of SIF cells) or contain very large catecholamine granules (type II) similar to chromaffin cells. Sub-type II of SIF cells is more frequently localized near blood vessels (Langley and Grant, 1999). SIF cells are located in the paravertebral sympathetic ganglia, adrenal glands and paraganglia (Unsicker, Habura-Fluh et al., 1978; Le Douarin and Kalcheim, 1999).

1.4.2 Migration routes of sympathoadrenal progenitors

The sympathoadrenal progenitors delaminate from the neural tube and migrate ventrally to the vicinity of the dorsal aorta. Under the influence of BMPs secreted by the dorsal aorta, the sympathoadrenal cells acquire catecholaminergic features. In the chick model, the adrenergic phenotype is eminent at embryonic day 3 (Ernsberger, Esposito et al., 2005)

It has been suggested that one part of this cell population re-migrates dorsally to form the primary and secondary sympathetic ganglia and the other part migrates more ventrally to form the chromaffin cells of the adrenal medulla.

Chicken adrenal glands do not form a proper “medulla and cortex structure” as it is known for mammals. The avian chromaffin and cortical cells are intermingled (Le Douarin and Kalcheim, 1999).

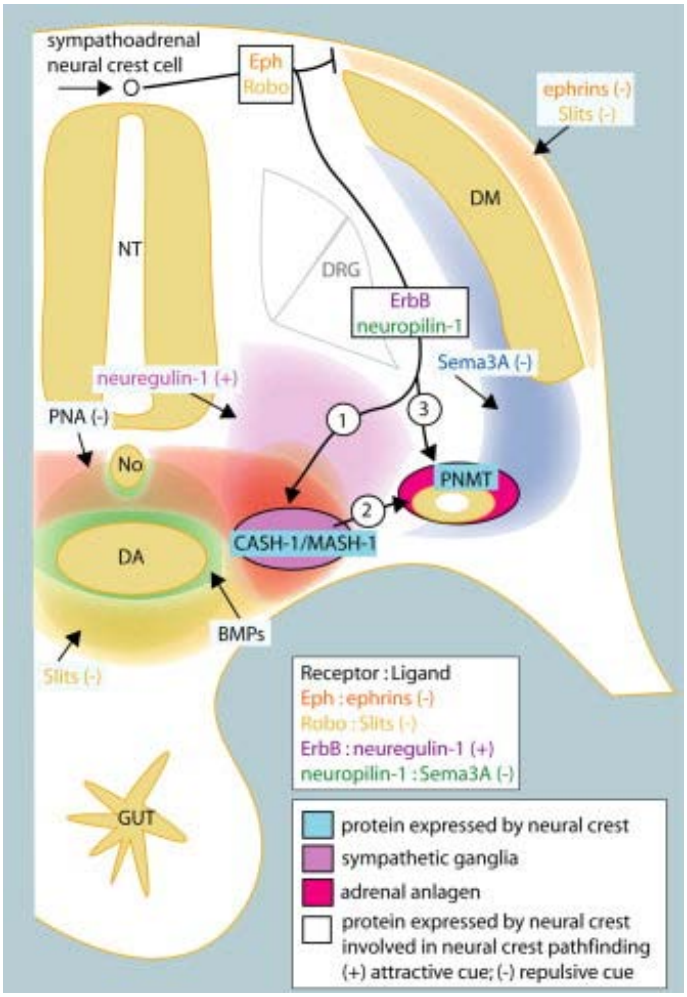


Fig 1.5: Scheme of patterning of sympathoadrenal neural crest (Harris and Erickson, 2007).

1.5 Determination of neuronal versus chromaffin phenotype

This chapter describes two different points of view on the cell fate determination of chromaffin cells of adrenal medulla and sympathetic neurons of sympathetic ganglia. The classical model suggests a common sympathoadrenal progenitor for both cell types and is based on the roles of glucocorticoids and NGF *in vitro* for determining chromaffin and sympathetic phenotypes, respectively. More recently, another model has been discussed, which is based on heterogeneity among sympathoadrenal cells before they reach their final destinations. This model suggests a putative pre-specification among premigratory or early migrating neural crest cells.

The scheme depicted in the Figure 1.6 summarizes the models of lineage relationship between sympathetic neurons and chromaffin cells. The classic model describes the

development of chromaffin cells and sympathetic neurons from a sympathoadrenal bipotential precursor. The alternative model suggests the possible existence of two distinct precursor cells, the chromaffin and neuronal precursors, respectively (Huber, 2006).

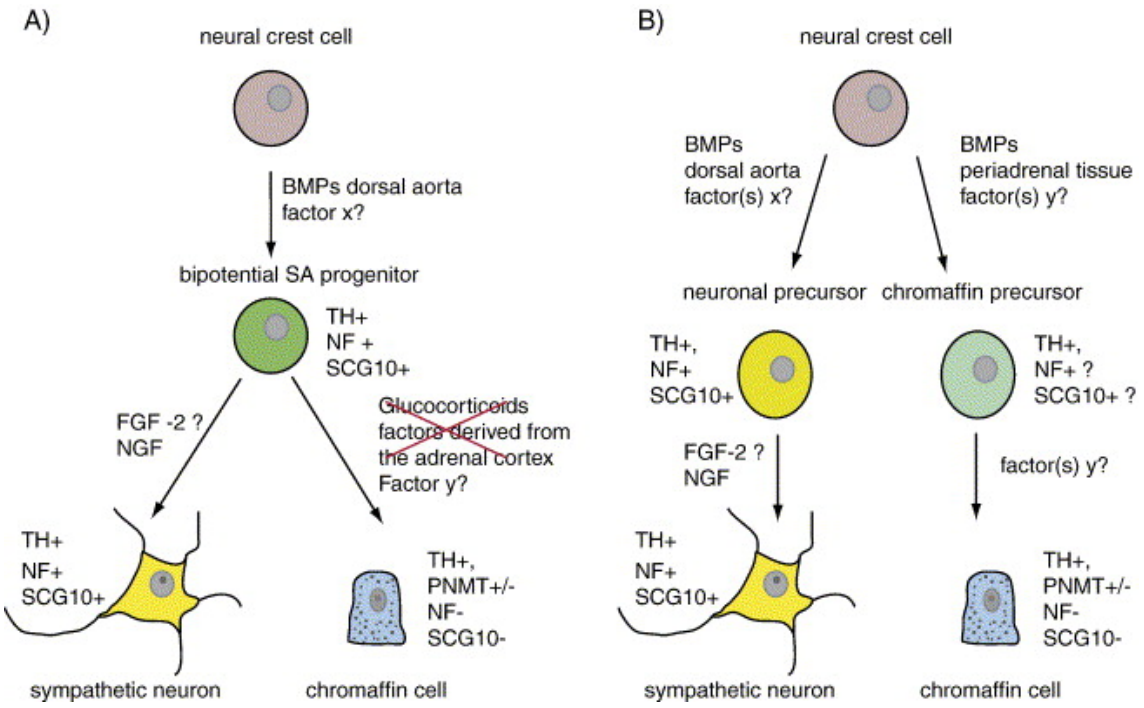


Fig 1.6: The lineage relationship between sympathetic neurons and chromaffin cells (Huber, 2006).

1.5.1 Hypothesis of a common sympathoadrenal progenitor lineage for sympathetic neurons and chromaffin cells

For several decades, it has been believed that the development of the sympathoadrenal cell lineage is well understood. The classic perception suggests a common neuroendocrine progenitor for both sympathetic neurons and chromaffin cells. Further, it has been is described that BMP signals from the dorsal aorta direct NC cells toward the sympathoadrenal fate and that subsequently, the different environments of adrenal gland or secondary sympathetic ganglia play crucial roles in the final differentiation steps of adrenomedullary cells and sympathetic neurons, respectively (Huber, 2006).

Based on *in vitro* data, there is ample evidence that supports the hypothesis of a common sympathoadrenal precursor. It has been described that SA cells are

bipotential neuroendocrine precursors that are able to produce both sympathetic neurons and adrenal medullary cells under the influence of environmental factors, NGF or glucocorticoids. It was widely assumed that the levels of NGF and glucocorticoids could determine the final fate of SA cells (Unsicker, Krisch et al., 1978; Doupe, Landis et al., 1985). Upon differentiation, the markers typical for chromaffin cells are downregulated in sympathetic neurons and neuronal markers are downregulated in chromaffin cells (Anderson and Axel, 1986; Anderson, Carnahan et al., 1991). Previous evidence underlining the existence of a common SA progenitor includes a transdifferentiation capacity of adrenal medullary cells. When dissociated rat chromaffin cells are exposed to NGF they start to extend neurites. This NGF-induced fiber outgrowth may be abolished by physiological concentrations of glucocorticoids that are *in vivo* produced by the adrenal cortex (Unsicker, Krisch et al., 1978). Another experiment confirmed the plasticity of chromaffin cells in response to environmental factors and the NGF-induced conversion of adrenomedullary cells into sympathetic neurons. This finding suggests that early chromaffin cells retain the properties of SA progenitors (Doupe, Landis et al., 1985) and can be channelled into the neuronal lineage.

1.5.2 Glucocorticoid hypothesis

In vitro studies have suggested that glucocorticoids and NGF levels determine the fate of SA cells. The survival and the maintenance of chromaffin cell features have been shown to depend on glucocorticoids (Doupe, Landis et al., 1985). It has been suggested that the differentiation of chromaffin cells depends on glucocorticoids provided by adrenal cortical cells. Glucocorticoids have been described to promote the induction of the adrenaline-synthesizing enzyme PNMT and suppress neuronal features (Anderson, 1993; Unsicker, 1993).

The hypothesis that glucocorticoids are important factors in SA cells differentiation into chromaffin cells has been challenged as genetically manipulated mice lacking the glucocorticoid receptor (GR) develop chromaffin cells. These chromaffin cells revealed the typical ultra-structural characteristics. However, the expression of PNMT and secretogranin-II has been impaired in the GR mutant mice (Finotto, Krieglstein et al., 1999; Huber, Combs et al., 2002). A recently performed study has supported the essential role of glucocorticoid signalling in chromaffin cell survival but not in chromaffin cell differentiation (Parlato, Otto et al., 2008).

The hypothesis of an essential role of glucocorticoid signalling in chromaffin cell differentiation has been also questioned by studies with SF1 mutant mice. Mice deficient for the nuclear orphan receptor steroidogenic factor-1 (SF1) lack the source of glucocorticoids - the adrenal cortical cells. These mice surprisingly generate cells with a typical chromaffin phenotype. Despite the absence of a cortex, the adrenomedullary cells are correctly located and have all the typical ultrastructural features of chromaffin cells. The numbers of NC cells colonizing the adrenal glands is smaller in comparison to wild type controls as shown by the expression of SOX10, a marker for undifferentiated NC cells, Phox2b, an early SA marker, and TH (Gut, Huber et al., 2005). The induction and expression of PNMT enzyme is impaired similar to the situation in GR-mutant mice (Finotto, Krieglstein et al., 1999; Gut, Huber et al., 2005).

1.5.3 Heterogeneity of sympathoadrenal cells

Sympathetic neurons and chromaffin tissue are characterized by expression of several markers that are common for both cell types. These markers are the neural crest marker HNK1, and markers for catecholaminergic features as Phox2B and TH. Sympathetic neurons, opposite to chromaffin cells, are characterized by pan-neuronal markers such SCG10 and neurofilament-M (NF-M). The presence of large chromaffin granules is a feature of neuroendocrine adrenomedullary cells. Ernsberger and colleagues have analysed the expression of neuronal and adrenal markers in different stages of avian development. They have shown the presence of TH-negative, Phox2B-positive catecholaminergic progenitors with very low mRNA levels of the neuronal marker NF-M in the presumptive regions of adrenal gland in early embryonic stages before the discrete adrenal gland is formed. The *in situ* hybridisation analysis has revealed the heterogeneity of the sympathoadrenal cells before invading the adrenal medulla (Ernsberger, Esposito et al., 2005). These findings have generated a new hypothesis supporting the model of pre-specification of the neural crest cell population (Harris and Erickson, 2007). The heterogeneity among SA cells suggests that sympathetic neurons and chromaffin cells might represent two distinct populations with predetermined fate in early phases of NC migration or even earlier.

1.6 Molecular determination of sympathoadrenal cell development

1.6.1 Transcriptional regulation of SA cell differentiation

Along their migratory pathways, the SA cells are exposed to several environmental extrinsic signals and cell-intrinsic factors. The sympathoadrenal precursors aggregating at the vicinity of the dorsal aorta are positive for Sox10, HNK1, and p75. Under the exposure of BMP signals subsequent expression of specific autonomic markers begins. The dorsal aorta-derived BMP signals have been characterized as BMP2 and BMP4 in chicken. The BMPs initiate expression of Ascl1 (the vertebrate homolog of Drosophila achaete scute complex proneural genes; Mash1-mouse homolog; Cash1-chicken homolog), Phox2a/b (the paired homeodomain transcription factor), dHand (the bHLH transcription factor) and Gata2/3 (Zinc-finger transcription factor), which trigger transcription of TH and dopamine-β-hydroxylase (DBH). Upon further differentiation, sympathetic neurons express SCG10 and neurofilament, which are general neuronal genes. The chromaffin cells become positive for chromogranins (Le Douarin and Kalcheim, 1999).

The scheme depicted in Figure 1.7 shows the relationship between the transcriptional regulations in the SA cell lineage (Huber, 2006).

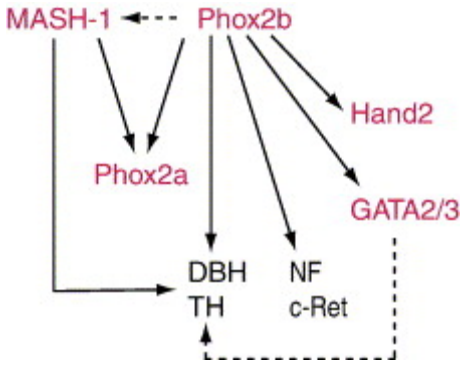


Fig 1.7: Transcriptional regulation of the development of SA cells (Huber, 2006).

1.6.2 Bone morphogenetic proteins

Bone morphogenetic proteins (BMPs) are growth factors that belong to the transforming growth factor β (TGFβ) superfamily. They have been implicated in many

different functions in vertebrate embryonic development and postnatal life (Chen, Zhao et al., 2004). BMP4 and BMP7 produced by the wall of dorsal aorta have been shown to trigger a sympathetic adrenergic phenotype in neural crest derived cells that have reached the vicinity of dorsal aorta (Ernsberger, Patzke et al., 1995; Ernsberger, Reissmann et al., 2000). These cells start to express transcription factors Phox2B/A, CASH1, Hand2, Gata2/3 and TH (Reissmann, Ernsberger et al., 1996). The inhibition of BMP signalling by noggin, a secreted polypeptide, blocks the expression of TH and DBH (Schneider, Wicht et al., 1999).

BMP4 expression has been detected in chicken adrenal glands but not in sympathetic ganglia. The initial BMP signals have been shown to trigger the expression of catecholaminergic markers in sympathoadrenal cells but persistent expression blocks the terminal neuronal differentiation characterized by extension of neurites. It has been shown that persistent BMP4 expression in sympathetic ganglia may increase the numbers of chromaffin granules in the 25% chromaffin cells, but is not sufficient to change the fate of sympathetic neurons toward a chromaffin cell fate. The proportion of these chromaffin cells within sympathetic ganglia has not been increased under the overexpression of BMP4 (Huber, Franke et al., 2008).

1.6.3 Mash1

The early studies of Mash1 deficient mice presented severe impairment in sympathetic neuron development and almost no changes in differentiation of adrenomedullary cells (Guillemot, Lo et al., 1993). However, a more profound analysis of Mash1 knockout mice revealed the role of Mash1 in the development of chromaffin cells. The majority of chromaffin cells displayed a very immature phenotype. The expression of TH was impaired; however, the Phox2B and Hand2 expression appeared unaltered. The chromaffin granules were absent from most of the chromaffin cells suggesting that Mash1 plays an important role in catecholaminergic differentiation and in the development of general adrenomedullary features (Huber, Bruhl et al., 2002).

1.6.4 Phox2B/A

Phox2B is expressed by peripheral autonomic and enteric neurons and by neuroendocrine chromaffin cells. The analysis of Phox2b and Mash1 mutant mice revealed differences in the phenotype of chromaffin cells between these two mutants.

The chromaffin cells of Phox2B deficient mice showed an even more immature phenotype and failed to express Phox2A, dHand and TH. Thus, the early development of chromaffin cells essentially depends on transcription factor Phox2B (Huber, Karch et al., 2005).

1.6.5 Insm1

Recently, the insulinoma-associated 1 gene (Insm1) that encodes a DNA-binding protein with five zinc-finger domains has been implicated in the development of the SA cell lineage. Insm1 acts as a downstream target gene of Mash1 and Phox2B during SA cell development. In Insm1 deficient mice, development and maturation of sympathetic neurons is largely unaltered, but the secondary sympathetic ganglia appear smaller. The differentiation of chromaffin cells is more severely affected. The Mash1 expression is upregulated whereas the expression of catecholaminergic markers (TH, DBH, PNMT) is downregulated suggesting that Insm1 plays a crucial role in the terminal differentiation of chromaffin cells (Wildner, Gierl et al., 2008).

1.6.6 dHand

After the precursors are specified to become sympathetic neurons, the expression of dHand (Hand2) transcription factor starts in sympathetic ganglion primordia (Howard, Stanke et al., 2000). Hand2 has been shown as a necessary factor for the survival of fetal and neonatal mouse sympathetic neurons, which depend on target-derived NGF (Doxakis, Howard et al., 2008).

1.6.7 Gata2/3

The expression of Gata2 starts after the onset of Mash1, Phox2B/A, and Hand2 expression but before the transcription of TH and DBH is activated. Gata2 (for chick) and Gata3 (for mouse) play an essential role for generation and differentiation of sympathetic neurons (Tsarovina, Pattyn et al., 2004). The analysis of Gata3 mutant mice confirmed the essential role of Gata3 for differentiation and survival of SA cells (Moriguchi, Takako et al., 2006).

1.7 The role of Notch signalling in the development of the peripheral nervous system

Notch and its ligands Delta or Jagged/Serrate are transmembrane proteins involved in cell-to-cell communication. Notch/Delta signalling is involved in a great number of developmental processes, involving the mechanisms of cell-fate specification, asymmetric cell division or lateral inhibition. More data are available about the involvement of Notch/Delta signalling in the development of the central nervous system than of the peripheral nervous system (Yoon and Gaiano, 2005; Kageyama, Ohtsuka et al., 2008; Tsarovina, Schellenberger et al., 2008).

The Notch receptor is activated upon binding of its ligand (Delta) that is expressed by neighbouring cell. Ligand binding results in proteolytic cleavage of Notch and subsequent release of the Notch intracellular domain (NICD). The NICD is translocated into the nucleus, where it regulates the transcription of several target genes, including members of the HES family of basic helix-loop-helix transcriptional repressors (Levy, Lah et al., 2002; Bray, 2006). HES1 induced by Notch signalling inhibits Mash1 expression in sympathetic neurons (Radtke and Raj, 2003).

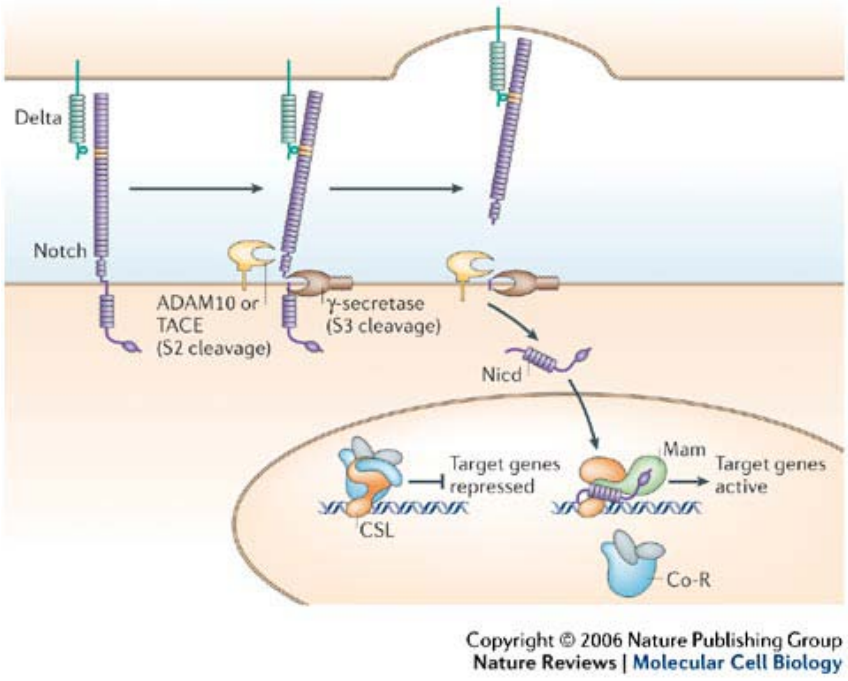


Fig 1.8: The Notch/Delta signalling (Bray, 2006).

The role of Notch/Delta signalling has been recently connected with the developmental processes of the peripheral nervous system, neurons and glial cells of dorsal root ganglia and sympathetic ganglia (SG), respectively. Active Notch signalling negatively

regulates neuronal differentiation and prevents the neighbouring cells from accepting the same fate by the mechanism of lateral inhibition and asymmetric cell division. Notch/Delta activity has been shown to play a role in the segregation of glial versus neuronal fates. In avian dorsal root ganglia, the differentiated neurons were positive for Delta1, a Notch ligand, whereas the cells positive for Notch1 and committed to glial fate were prevented from neuronal differentiation (Wakamatsu, Maynard et al., 2000). The analysis of Notch signalling by gain- and loss-of-function experiments in chicken confirmed previous results. It has been shown that activated Notch signalling decreased the number of neurons whereas inhibition of Notch increased the portion of neurons in sympathetic ganglia. The Notch signalling is involved in the neurogenesis of sympathetic and sensory neurons and early ganglia formation (Tsarovina, Schellenberger et al., 2008).

The expression pattern of Delta-like 4 during chicken development has been studied. The analysis has shown the DLL4 expression in embryonic blood vessels, heart, somites, neural tube, eye, limb and paraaortic bodies (Nimmagadda, Geetha-Loganathan et al., 2007).

1.8 Vascular pattern of endocrine organs

Sympathoadrenal cells give rise to two different types of derivatives: neuroendocrine cells of adrenal medulla and sympathetic neurons (Le Douarin and Kalcheim, 1999). The adrenal gland, in contrast to the sympathetic ganglia, belongs to the endocrine organs that have a big demand in the blood supply as they liberate hormones into the circulation. Chromaffin cells and the SIF cells type II (that closely resemble chromaffin cells) are mostly found in very close proximity to the blood vessel (Langley and Grant, 1999).

The formation of blood vessels by proliferation and differentiation of angioblasts is called vasculogenesis. The term angiogenesis means sprouting and growth of already present vessels. Several growth factors are involved in the angiogenic growth, such as vascular endothelial growth factor (VEGF), Ephrins, transforming growth factor β (TGF-β), fibroblast growth factor (FGF) (Nimmagadda, Geetha-Loganathan et al., 2007). Recently, endocrine gland-derived vascular endothelial growth factor (EG-VEGF) was identified as the first tissue-specific angiogenic molecule (Ferrara, LeCouter et al., 2002; LeCouter, Lin et al., 2002; LeCouter, Lin et al., 2002).

2 OBJECTIVES

The neuroendocrine chromaffin cells and sympathetic neurons, both neural crest derivatives, are supposed to originate from a common lineage, the so-called sympathoadrenal lineage. Sympathoadrenal progenitor cells delaminate from the neural tube; migrate along the ventral pathway, aggregate in the vicinity of the dorsal aorta where they, under the control of BMPs, start to express tyrosine hydroxylase. Recently, gene expression analysis have shown heterogeneity among sympathoadrenal cells at the site of the dorsal aorta before the cells invade their final destinations, sympathetic ganglia and adrenal glands, respectively. These data challenge the classical concept of sympathoadrenal cell lineage and suggest that specification of sympathetic neurons and chromaffin cells might occur early in NC development before the NC cells invade their final destinations.

I attempt to answer following questions in this project:
Do sympathetic ganglia and adrenal chromaffin cells share a common sympathoadrenal progenitor, or do their progenitors have distinct fates already at the level of the neural tube?
Does the Notch/Delta signalling play a role in the development of the sympathoadrenal lineage?
Does the vascular pattern differ in developing adrenal anlagen and sympathetic ganglia, respectively?

In order to investigate whether progenitors of sympathetic neurons and chromaffin cells are prespecified already within the neural tube the *in ovo* electroporation technique were used. Hemitube electroporations of GFP-constructs were performed at various developmental stages of crest delamination. I then established the technique of single crest cells electroporations using GFP-constructs at embryonic day (E) 2. Immunocytochemical analysis of GFP-labelled derivatives was then performed at E6 when the adrenal gland and sympathetic ganglia represent defined anatomical structures. We expected to find GFP/TH co-labelled cells in both the adrenal anlagen and the sympathetic ganglia, respectively, or in one of these two locations only.
In the second part of this project, I investigated the expression pattern of several Notch/Delta signalling members.
In the last part of this thesis I studied the vascular pattern within developing adrenal gland and sympathetic ganglia.

3 MATERIALS AND METHODS

MATERIALS

3.1 Experimental animals

Fertilized quail (*Coturnix coturnix Japonica*) and chicken (*Gallus gallus*) eggs of strains "Tetrabraun" and "Leghorns" from commercial sources (LSL RHEIN-MAIN, Dieburg, GE) were used for *in vitro* and *in vivo* experiments.

3.2 Laboratory material

Barrier tips	Avant Guard, USA
Eppendorf tubes	Eppendorf, GE
Falcon tubes	Becton Dickinson Labware, USA
Laboratory glassware	Brand, GE
Microscope cover glass	R. Langenbrinck, GE
Microscope Superfrost slides	R. Langenbrinck, GE
Needle 21G, 40mm	Braun, GE
Pipette tips	Greiner Bio-one, GE
Sterile pipettes	Constar, USA
Surgical tules	F.S.T., GE
Syringe	Becton Dickinson Labware, USA

3.3 Chemicals, reagents and solutions

3.3.1 General chemicals and reagents

Acetone	Zentrallager INF, University Heidelberg
Aqua ad iniectabilia	Braun, GE
Distillate water	Milipore (MiliQ Water purification system)
EtOH 70%, 85%, 90%, 100%	Zentrallager INF, University Heidelberg
Xylene	Zentrallager INF, University Heidelberg

3.3.2 Reagents for molecular biology

GenElute Plasmid Mini prep Kit	Sigma, USA

GenElute Plasmid Midi prep Kit	Sigma, USA
2-Log DNA Ladder	New England Biolabs, USA

3.3.3 Buffers and media for molecular biology

50x TAE buffer

242g Tris (Roth, GE)

57,1ml acetic acid

100ml 0,5 M EDTA (pH 8; Merck, GE)

LB-medium (Q-biogene, USA)

10g Trypton

5g Yeast-Extract

10g NaCl

15g Agar

ad 1 l distillate water

pH 7,0; autoclave

LB/AMP-medium

100µg/ml Ampicilin (Sigma, USA) in LB-medium

LB/Agar-medium (Q-biogene, USA)

10g Trypton

5g Yeast-Extract

10g NaCl

15g Agar

ad 1 l distillate water, autoclave

AMP-Agar-Plates for blue/white colony screening

LB/Agar-medium (Q-biogene, USA) with 100µg/ml Ampicilin (Sigma, USA), 0,5 mM IPTG (Sigma, USA), and 80µg/ml X-Gal (AppliChem, GE)

Collect the white colonies only. Blue/white colony screening assay is used to distinguish recombinant (white) colonies among non-recombinant (blue) ones.

3.3.4 Plasmids

For electroporation following plasmids were used:

Name of plasmid	Size	Notes
pCAGGS-AFP	5,54kpb	constructed by Hidesato Ogawa (Momose, Tonegawa et al., 1999)
pT2K-CAGGS-EGFP	8.9kpb	(Sato, Kasai et al., 2007)
pCAGGS-T2TP	7kpb	(Sato, Kasai et al., 2007)

Table 3.1: Plasmids for electroporation.

For *in situ* hybridisation probe synthesis the following plasmids were used:

Name of plasmid	Name of the probe	Abbreviation	Source	Antisense	Sense
pBS Neurofilament-M	Chick(c) NF-M	NF-M	Dr. Uwe Ernsberger	EcoRI, T7 Poly	HindIII, T3 Poly
pBS Delta-like 1	cDll 1	Dll 1	David Ish-Horowitcz	EcoRI, T3 Poly	no info
pBS Delta-like 4	cDll 4	Dll 4	BBSRC chicken EST	NotI, T3 Poly	EcoRI, T7
pBS Hairy 1	cHairy 1	Hairy 1	Isabel Palmeirim	HindIII, T7 Poly	no info
pBS Hairy 2	cHairy 2	Hairy 2	Isabel Palmeirim	HindIII, T7 Poly	no info

Table 3.2: Probes for *in situ* hybridisation riboprobes synthesis.

Chicken cDNA for NF-M kindly provided by Uwe Ernsberger was used for riboprobe synthesis (Schneider, Wicht et al., 1999).
Plasmids for Dll1, Dll4, Hairy 1, and Hairy 2 were kindly provided by Prof. Dr. Chaya Kalcheim, Jerusalem.

3.3.5 Reagents for *in situ* hybridisation

TE-buffer (Tris/EDTA buffer)

1,21g Tris (Roth, GE)

0,372g EDTA (Merck, GE)

ad 1 l distillate water

pH 8

Box-buffer

50% formamide (J.T.Baker, NE)

10% 20xSSC

40% Aqua ad iniectabilia (Braun, GE)

20x SSC (sodium chloride-sodium citrate)

175,3g NaCl (J.T.Baker, NE)

88,2g tri-natriumcitrat ($C_6H_9Na_3O_9$; AppliChem, GE)

1,3ml HCl (J.T.Baker, NE)

pH 7

Hybridisation buffer

900µl distillate water

1ml 10xSalt

100µl 50x Denhardts (Sigma, USA)

1ml yeast-RNA (10mg/ ml; Sigma, USA)

2ml 50%Dextran sulfate (Roth, GE)

5ml formaldehyde (Merck, GE)

10x Salt

7,7g Tris (Roth, GE)

53,9g NaCl (J.T.Baker, NE)

3,55g NaH_2PO_4 (AppliChem, GE)

4,45g Na_2HPO_4 (AppliChem, GE)

50ml 0,5M EDTA (Merck, GE)

30ml 1M HCl (J.T.Baker, NE)

ad 500ml distillate water

pH 7,5

MAB

11,6g maleic acid (Sigma, USA)

8,75 NaCl (J.T.Baker, NE)

7,5g NaOH (J.T.Baker, NE)

ad 1 l distillate water

pH 7,5, autoclave

MABT

MAB with 0,1% Tween (Sigma, USA)

AP-buffer

40ml 1M Tris (pH 9,5; Roth, GE)

20ml 1M $MgCl_2$ (Merck, GE)

8ml 5M NaCl (J.T.Baker, NE)

96mg Levamisol (Sigma, GE)

400µl Tween (Sigma, USA)

ad 400ml distillate water

3.3.6 Reagents for immunostaining

DAPI	Boehringer, GE
Horse serum	Gibco-Invitrogen, GE
Goat serum	Gibco-Invitrogen, GE

Blocking solution

0,5% Triton-X-100 (Merck, GE)

1% BSA

1xPBS

Citrate buffer for antigen retrieval

18ml 0,1M citric acid (AppliChem, GE)

82ml 0,1M sodium citrate dehydrate (J.T.Baker, NE)

ad 1 l water distillate

pH 6

1x Phosphate buffered saline (PBS)

8g NaCl (J.T.Baker, NE)

0,2g KCl (Merck, GE)

1,15g Na_2HPO_4 x 2 H_2O (AppliChem, GE)
0,2g KH_2PO_4 (Merck, GE)
ad 1 l water distillate

0,1 M phosphate buffer
2,62g NaH_2PO_4 (Merck, GE)
14,41g Na_2HPO_4 x 2 H_2O (AppliChem, GE)
ad 1 l water distillate

4% paraformaldehyde (PFA)
40g PFA
ad 1 l 0,1 M phosphate buffer

PBS serum
10% horse serum (HS) in PBS

Mowiol
2,4g Mowiol (Calbiochem, USA)
6g Glycerol (J.T.Baker, NE)
6ml Distilled water
12ml 0,2M Tris (pH 8,5; Roth, GE)

3.3.7 Primary antibody

Antibody	**Dilution**	**Source**
rabbit anti-GFP	1:200	Molecular Probes-Invitrogen, USA
mouse anti-TH	1:400	Chemicon (Milipore), GE
mouse anti-HNK1	1:200	BD Pharmingen, GE
mouse anti-QH1	1:200	Developmental Studies Hybridoma Bank, USA

Table 3.3: Primary antibody.

3.3.8 Secondary antibody

Antibody	Dilution	Source
Alexa Fluor 555 goat anti-mouse IgM	1:500-1:1000	Molecular Probes-Invitrogen, USA
Alexa Fluor 488 goat anti-mouse IgG1	1:500	Molecular Probes-Invitrogen, USA
DyLight 488-conjugated donkey anti-rabbit IgG	1:500	Jackson ImmunoResearch, USA
Cy2™-conjugated anti-rabbit	1:200	Jackson ImmunoResearch, USA
Biotinylated rabbit anti-mouse	1:200	Jackson ImmunoResearch, USA
Cy3™-conjugated anti-mouse	1:400	Jackson ImmunoResearch, USA

Table 3.4: Secondary antibody.

3.3.9 Reagents for *in ovo* transfection

Plasmid solution for electroporation

Plasmid was diluted with sterile 1xPBS to desired concentration; several grains of fast green powder (AppliChem, GE) were added to reach the green colour of plasmid solution for better visualization upon electroporation.

Ink solution

The ink-diluted 1:100 with sterile 1xPBS was injected under the blastoderm before electroporation to increase the contrast. At this dilution the survival of embryos was not altered.

Calcium-magnesium-free Hanks' Balanced Salt Solution (Ca/Mg-free HBSS)

50ml HBSS (10x; Gibco-Invitrogen, GE)
2,5ml NaHCO3 (7,5%; Gibco-Invitrogen, GE)
ad 500ml Aqua ad iniectabilia, autoclave

CMF/ATB solution

Ca/Mg-free HBSS with mix of antibiotics Penicilin/Streptomycin/Neomycin (1:100; Gibco-Invitrogen, GE).

3.4 Instruments

Analytical scales	Sartorius, GE
Camera AxioCamMR3	Zeiss, GE
Centrifuge	Eppendorf, GE
Cold light source	Zeiss, GE
Cycler	Eppendorf, GE
ECM® 830 electroporator	BTX Harvard Apparatus, USA
Electrode holder	F.S.T., GE
Magnetic heating mixer	Heidolph, GE
Magnetic stands	F.S.T., GE
Magnetic steel base	Merck, GE
Micromanipulatores left, right	F.S.T., GE
Microscope Zeiss Axiophot	Zeiss, GE
Microtome	Microm, GE
Nanoliter 2000	WPI, USA
Personal computer	Transtec, USA
pH-meter	Knick, GE
Puller PC-10	Narishige, Japan
Refrigerators 4°C, -20°C, -80°C	Liebherr, GE and Revco, USA
Stereomicroscope Zeiss Discovery V8	Zeiss, GE
Tungsten wire-electrodes	self-made
Vortex	Heidolph, GE
Water bath	Julabo, GE

3.5 Software

Zeiss AxioVision	Zeiss, GE
GraphPad, Prism	GraphPad, Software, USA
Photoshop 5.0	AdobeSystems, USA

METHODS

3.6 Animal handling

Chicken and quail embryos were incubated till desired stages and staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1992). Somite number, limb bud development, and visceral arches served as the main characteristics of developmental stage. The incubation conditions were the following: temperature 37-38°C and high humidity ensured by the reservoir of water in the incubator. For GFP-transfection experiments fertilized chicken eggs were incubated till E2. Before electroporation, every egg was cleaned with a towel wetted by 70% ethanol. To prevent the leakage of albumin when opening the egg, one to three millilitres of albumin were taken out by a 21G needle and syringe. The small hole made by the syringe was sealed by melted paraffin. A window (diameter *circa* 20 mm) in the eggshell was done above an embryo by scissors. In order to visualize a transparent embryo, the ink solution was injected beneath the blastoderm. Thereby the contrast between the embryo and the yolk was increased and the stage of the embryo was easily determined. Vitelline membrane covering the yolk and the embryo was cut out above the area of manipulation. *In ovo* transfection was performed. After transfection, several drops of sterile HANKS+ATB solution were added to the embryo. The eggshell was tightly sealed with the Tesa-tape, the egg was marked and the embryo was reincubated till E6.

3.7 Molecular biology methods

3.7.1 Transformation of plasmid into competent bacteria

We used two lines of competent bacteria, E. coli TOP10 or E. coli DH5-α. The bacteria were slowly defrosted from -80°C. The mixture of 2µl of plasmid and 5µl of bacteria were kept on ice for 30min. The heat shock followed: 45s in 42°C water bath, then 1-2 min on ice. After heat shock 450µl of LB-medium was added and the bacteria solution was kept for one hour in a 37°C water bath. The solution was pipetted up and down and the Ampicilin-Agar-plate was inoculated with 50µl. The plate was incubated O/N at 37°C.

3.7.2 Plasmid purification

Transformed competent bacteria E. coli were cultivated O/N in 5ml (for Mini Prep Kit, Sigma) or 50ml (for Midi Prep Kit, Sigma) of LB/AMP-medium at 37°C under constant shaking. Next day, the plasmid was isolated according to the manufacturer's instructions. The plasmid was eluted with Aqua ad iniectabilia (Braun).

The plasmid concentration was measured photometrically. The purity of the plasmid was assessed by the ratio of absorbance measurement at 260nm and 280nm (OD260/280). The value of OD260/280 of clean product (with water as solvent) is between 1,8 and 2,0. Values lower than 1,8 indicate contamination of the product by proteins.

The plasmid solutions were stored at -20°C.

3.7.3 ISH-probe synthesis

Digoxigenin (DIG)- labelled probes were used for ISH.

cDNA plasmid was linearized by appropriate restriction enzymes. The probes for ISH were synthesized by *in vitro* transcription from linearized cDNA plasmids with appropriate RNA polymerases, T3-polymerase and T7-polymerase, and DIG-labelling mix. The riboprobes were purified on mini Quick Spin RNA Columns according to the manufacturer ´s instructions (Roche). The riboprobes were diluted 1:10 in TE-buffer for *in situ* hybridisation

When no information about restriction enzymes and polymerase were available, the transcription templates were synthesized by PCR reaction with M13 (Roche) forward and reverse primers compatible for pBluescript.

3.8 Transfection of neural crest cells *in ovo*

We used different approaches for solving the question of possible SA progenitor cell heterogeneity at the level of the neural tube in the chicken model. For single-cell lineage analysis *in vivo* we used several approaches, including microinjection, ventral-to-dorsal electroporation (VD ep), and micropipette electroporation (Mep). Hemitube electroporation (HM ep) was used for labelling half of the neural tube.

3.8.1 Hemitube electroporation

HM ep allows labelling of all types of the NC progenitor cells within one half of the neural tube. Plasmid solution was injected into the lumen of the neural tube, positive and negative electrodes were inserted into the egg on the top of the yolk parallel to the embryo. Four electric pulses of 10ms length and voltage 28V in 100ms intervals were applied.

We performed GFP-labelling of neural tube at different time points of embryonic development from 15-somite stage till 25-somite stage of chicken embryos. Subsequently, we counted the numbers of GFP^+/TH^+ immunolabelled cells in the sympathetic ganglia vs. the adrenal glands at E6 (10µm paraffin cross section).

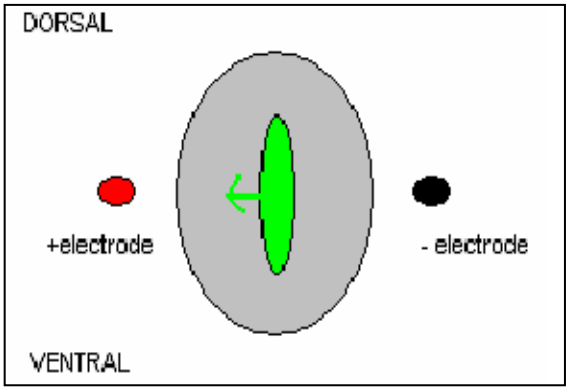


Fig 3.1: Scheme of hemitube electroporation. One half of the neural tube is transfected.

3.8.2 Ventral-to-dorsal electroporation

By VD ep several NC cells only in the dorsal midline of the NT were transfected. Plasmid solution was injected into the lumen of the neural tube. The negative electrode was inserted into the yolk beneath the embryo exactly below the neural tube. The positive electrode was placed on the dorsal midline of the neural tube; only the very sharp tip of the anode touched the neural tube. The prospective sympathoadrenal cells originate from the trunk neural crest between the levels of somite pairs 18^{th} till 24^{th}. The electroporation of the neural tube was limited to this area. Four electric pulses of 10ms and 28V in 100ms intervals were applied.

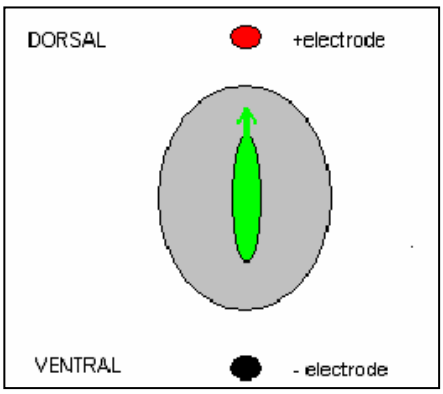


Fig 3.2: Scheme of ventral-to-dorsal electroporation. The cells in the dorsal midline of the NT are transfected.

3.8.3 Microinjection

Microinjection is a mechanical method for delivering a DNA solution into the cells without an application of electrical forces. We used Nanoliter 2000 (WPI, USA) to transfect single neural crest cells in the neural tube of the embryonic day 2 old chicken embryos. The axial level of transfection corresponded to the “sympathoadrenal” level of the neural tube.

For *in ovo* microinjection the chicken eggs were manipulated as described previously. Embryos were microinjected under the stereomicroscope (Zeiss, GE). A glass capillary was connected to the nanoliter injection device and filled automatically with GFP-plasmid solution. The plasmid pCAGGS-AFP at different concentrations (1µg/µl, 2µg/µl, and 4µg/µl) was used. The tip of the capillary entered the epidermis and was placed in the dorsal aspect of the neural tube. The plasmid solution of volumes 1µl up to 5µl was automatically delivered into the cells. The capillary was removed from the embryo and the embryo was re-incubated till E6.

3.8.4 Micropipette electroporation

In order to perform transfection of discrete neural crest populations, we modified a method of single-cell electroporation described by Haas and colleagues (Haas, Sin et al., 2001). This technique used the electric pulses from a DNA (GFP)-filled micropipette. The negative electrode (cathode) was inserted into the glass micropipette filled with the plasmid DNA solution, and the positive electrode (anode) was placed under the embryo into the yolk. The tip of the glass micropipette entered the ectoderm and touched the dorsal aspect of the neural tube. The square wave generator (BTX ECM830) delivered four pulses at 50V, and 15 ms long with the interval of 100ms. By

injecting the current, only the cell at the tip of the micropipette was transfected. The resistance of the glass micropipette after the electric pulse was 1,9 -2,0 MΩ.

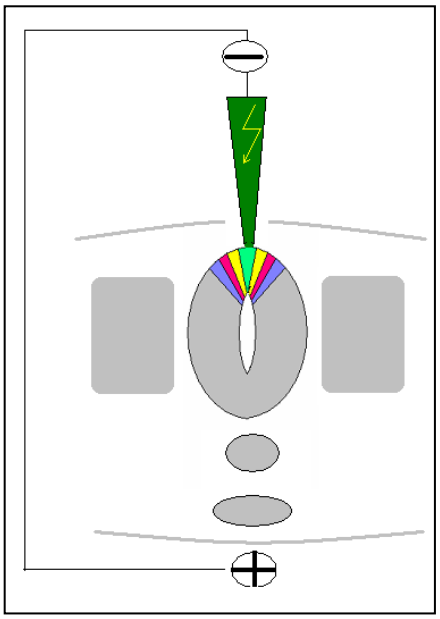


Fig 3.3: Scheme of micropipette electroporation.

3.8.5 Control of GFP-transfection

According to the GFP-vector used, the control of GFP expression was done three up to six hours (T_1) after the transfection for the first time and 20 up to 24 hours after the transfection for the second time (T_2). The Tesa-tape was removed using scissors and the embryo was analysed for GFP expression under the Zeiss stereomicroscope equipped with epifluorescence. The window in the eggshell was sealed again with Tesa-tape and the embryos were kept in the incubator till desired stages.

As a control of single cell electroporation, we fixed an equal number of embryos immediately after the first control of GFP-expression at T_1. These embryos were fixed in 4%paraformaldehyde in PBS and processed for an immunohistochemical analysis for GFP. The embryos incubated till embryonic day (E) 6 were fixed and processed for the immunohistochemistry of GFP and TH.

3.9 Histological methods

3.9.1 Tissue preparation for ISH and IHC

Embryos were dissected and decapitated at desired stages of development. Dissected embryos were washed twice in 1xPBS and fixed by 4% paraformaldehyde (PFA). After

fixation, the tissue was washed twice in 1xPBS and subsequently dehydrated in a graded series of ethanol-acetone and embedded in paraffin (see Paraffin embedding protocol for details). Tissue in paraffin blocks was cut into 10µm thin sections on microtome. Sections were mounted on Superfrost slides. Sections were deparaffinized and rehydrated before performing in situ hybridisation or immunohistochemistry staining.

Paraffin embedding protocol

2x washing in 1xPBS	5min	RT
Fixation in 4% PFA	O/N	4°C
2x washing in 1xPBS	5min	RT
1x washing in 70%EtOH	5min	RT
1x washing in 70%EtOH	O/N	4°C
2x washing in 80%EtOH	30min	RT
2x washing in 96%EtOH	30min	RT
3x washing in 100%EtOH	30min	RT
1x washing in 100%EtOH/Acetone (1:1)	30min	RT
2xwashing in Acetone	30min	RT
Paraffin I	30min	56°C
Paraffin I	90min	56°C

Paraffin embeding on paraffin embedding station.

Deparaffinisation and rehydration of the slides

2x washing in Xylene	10min	RT
2x washing in 100%EtOH	5min	RT
1x washing in 96%EtOH	5min	RT
1x washing in 70%EtOH	5min	RT
2x washing in 1xPBS	10min	RT

3.9.2 In situ hybridization

Non-radioactive *in situ* hybridization for chicken neurofilament-M (NF-M) mRNA was performed. *In situ* hybridisation on 10µm thin paraffin sections was performed with digoxigenin (DIG)-labelled antisense cRNA. The riboprobes were diluted 1:10 in TE-buffer for *in situ* hybridisation. The detection of DIG-probes was performed with the anti-DIG antibody coupled to alkaline phosphatase and 4-nitroblue tetrazolium chloride/

5-bromo-4-chloro-3-indolyl-phosphate (NBT/ BCIP) colour substrate. DIG-labelled sense riboprobes served as a control.

3.9.3 Immunohistology

Immunofluorescence for TH and GFP

Ten µm thick rehydrated paraffin sections were pre-treated with blocking solution and processed for double-immunostaining with antibodies against TH (mouse anti-TH, 1:400, Chemicon International, GE) and green fluorescent protein (rabbit anti-GFP, 1:200, Invitrogen, USA). Before applying the first antibody, the antigen retrieval was performed to uncover the epitope of TH antigen (see Antigen retrieval protocol). After overnight incubation with primary antibodies at 4°C, the sections were incubated with secondary antibodies. Cy3^{TM}-conjugated anti-mouse (1:500, Jackson ImmunoResearch, USA) was used as the secondary antibody for detection of TH. For detection of GFP the following secondary antibodies were used: Cy2^{TM}-conjugated anti-rabbit (1:200, Jackson ImmunoResearch, USA) or DyLight 488-conjugated donkey anti-rabbit IgG (1:500, Jackson ImmunoResearch, USA). Nuclei were counterstained with 4',6'-diamidino-2-phenylindole dichloride (DAPI, 1:10,000, Molecular Probes, USA). The sections were mounted with fluorescent-covering medium Mowiol.

Antigen retrieval

Deparaffinized slides in the glass cuvettte were immersed into the citrate buffer and inserted in the microwave. The buffer was brought to boiling at 900W. After boiling, the intensity of heating was switched to 270W and the buffer was heated for five minutes. Then the slides were rinsed with 1xPBS.

TH immunohistochemistry with colorimetric detection

The rehydrated slides were pre-incubated with 3% hydrogen peroxide (J.T.Baker, NE) in PBS for 30min. After pre-incubation the slides were incubated with the first antibody (to TH) for 30min. The washing step in 1xPBS followed. The secondary biotinylated horse anti-mouse antibody was applied for 30min. After secondary antibody incubation, sections were rinsed with 1xPBS and incubated for 60min with avidin and biotinylated horseradish-peroxidase-macromolecular complex (Vector Elite ABC Reagent, USA). Sections were rinsed and stained with 3-amino-9-athylcarbazol (AEC; Sigma, GE) for 30min. Three washing steps in 1xPBS for 10min each followed. Sections were mounted with glycerol gelatine (Merck, GE).

HNK1/QH1 immunostaining

Ten µm thick rehydrated paraffin sections of quail embryos were pre-treated with blocking solution and processed for double-immunostaining with antibodies against HNK1 (mouse anti-HNK1, 1:200, BD Pharmingen, GE) and QH1 (mouse anti-QH1, 1:200, Developmental Studies Hybridoma Bank, USA). After overnight incubation with primary antibodies at 4°C, and washing in 1xPBS, the sections were incubated with secondary antibodies. Alexa Fluor 555 goat anti-mouse IgM (1:500 or 1:1000; Molecular Probes-Invitrogen, USA) for anti-HNK1 antibody and Alexa Fluor 488 goat anti-mouse IgG1 (1:500, Molecular Probes-Invitrogen, USA) for detection of anti-QH1 antibody were used. Nuclei were counterstained with 4',6'-diamidino-2-phenylindole dichloride (DAPI, 1:10,000, Molecular Probes). The sections were mounted with fluorescent-covering medium Mowiol.

3.10 Microscopicy analysis

The electroporation procedures and subsequent control of GFP expression were performed under a Zeiss stereomicroscope (Ziess DiscoveryV8; Zeiss, GE) equipped with epifluorescence and filter for GFP. The *in ovo*-photographs were taken by a Zeiss camera with cooling system, the Zeiss AxioVision Software was used to set an exposure time. Pictures were assembled in Photoshop 5.0 (AdobeSystems).

The transverse paraffin sections were analyzed on a Zeiss Axiophot equipped with objectives 5x, 10x, 20x, and 40x and with a camera Zeiss Axiophot. The histological sections were photographed by Zeiss camera system and saved in JPG or TIFF format on the personal computer. Figures were assembled in Adobe Photoshop.

3.11 Cell number analysis

NF-M *in situ* hybridisation and TH immunohistochemistry

Cells expressing detectable levels of mRNA for NF-M (dark blue) and positive cells for TH-immunoreactivity (red) were counted in 10µm-thick paraffin transverse sections. The analysed area started at the rostral aspect of the adrenal gland and continued 140 µm caudally. The numbers of $TH^+/NF\text{-}M^-$ and $TH^+/NF\text{-}M^+$ cells were counted in every fifth section.

3.12 Statistical analysis

For statistical evaluation of the presence of chromaffin-like cells in SG, a one-way ANOVA followed by Newman-Keuls Multiple Comparison test was performed using the GraphPad Prism program.

4 RESULTS

The avian model has been used in our experiments. Its advantage rests in the availability of large numbers of eggs and in the fact that work with chicken embryos is not subjected to the regulations of the animal experimentation.
In order to distinguish neural crest cell fate determination at the level of the neural tube *versus* the target organ level two different approaches were employed: hemitube electroporation and single-cell labelling.
The chapter 4.1 describes hemitube electroporation, which determines the proportional distribution of NC derivatives within the pool of the emigrating NC cells as the function of the time of delamination. The chapter 4.2 contains the description and results of three direct methods of single NC progenitor labelling and its derivatives: ventral-to-dorsal electroporation, microinjection, and micropipette electroporation. The micropipette electroporation resulted in the highest efficiency among the employed methods of single-cell labelling. The results presented in the chapter 4.3 show the presence of chromaffin-like cells in sympathetic ganglia. Chapter 4.4 brings together the results of immunohistochemistry and *in situ* hybridisation analyses of single-cell labelled cells. The last two chapters describe preliminary data regarding the expression pattern of Notch signalling molecules (Chapter 4.5) and trunk vascular pattern during chicken embryogenesis (Chapter 4.6).

4.1 Hemitube electroporation experiments

We hypothesized that sympathetic neurons and chromaffin cells may originate from different progenitor lineages. These presumptive lineages might be determined already at the level of neural tube and they might have different timing for their emigration. One of our approaches to prove this hypothesis was hemitube electroporation. This method represented an indirect approach for proving the distribution of the NC cell derivatives within the pool of delaminating NC cells.

The emigration of neural crest cells from the NT starts at levels opposite to the epithelial somites and is organized in waves (Sela-Donenfeld and Kalcheim, 1999).

We GFP-labelled (pCAGGS-AFP) the hemitube of chicken embryos at different time points of embryonal development starting at 15-somite stage and continuing till 25-somite stage. When neural tube was electroporated at younger stages before the

neural crest cells have emigrated the neural tube, all derivatives of the neural crest were marked. When the embryos were electroporated at later stages, the early delaminating cells remained GFP-unlabelled as they were already out of the neural tube at the time of electroporation.

4.1.1 Control of hemitube electroporation

The electroporated embryos were checked for GFP expression three up to six hours at T_1=3-6hr (Figure 4.1) and 24 hours at T_2=20-24hr (Figure 4.2 and 4.3) after the transfection. The successfully electroporated embryos were kept in the incubator for another three days. At embryonic day six, the embryos were harvested and processed for paraffin embedding and immunohistochemistry. The sympathetic ganglia and the adrenal glands at E6 were analysed for GFP and tyrosine hydroxylase immunoreactivity.

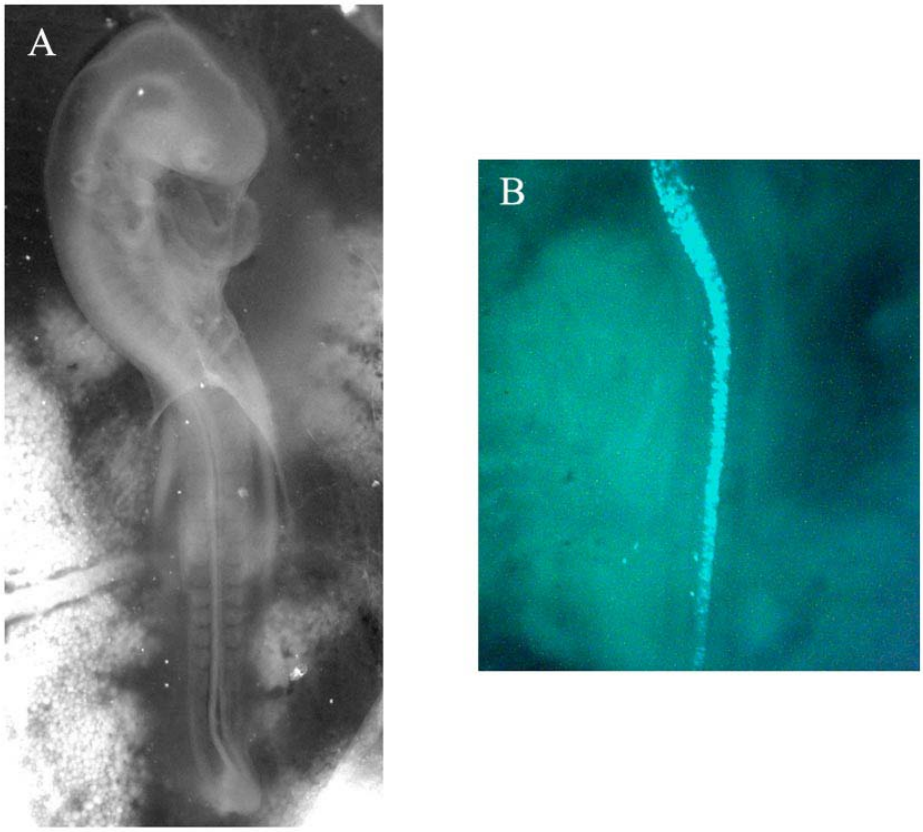


Fig 4.1: The first control of hemitube electroporation at T_1. (A) The bright field image: the dorsal view of embryo E2, 5 (HH stage 18). (B) *In vivo* image: dorsal view of electroporated chicken embryo made three hours after HM ep to control GFP-expression. Half of the neural tube shows a strong GFP-signal. The labelled neural crest cells are still present in the neural tube at this time point.

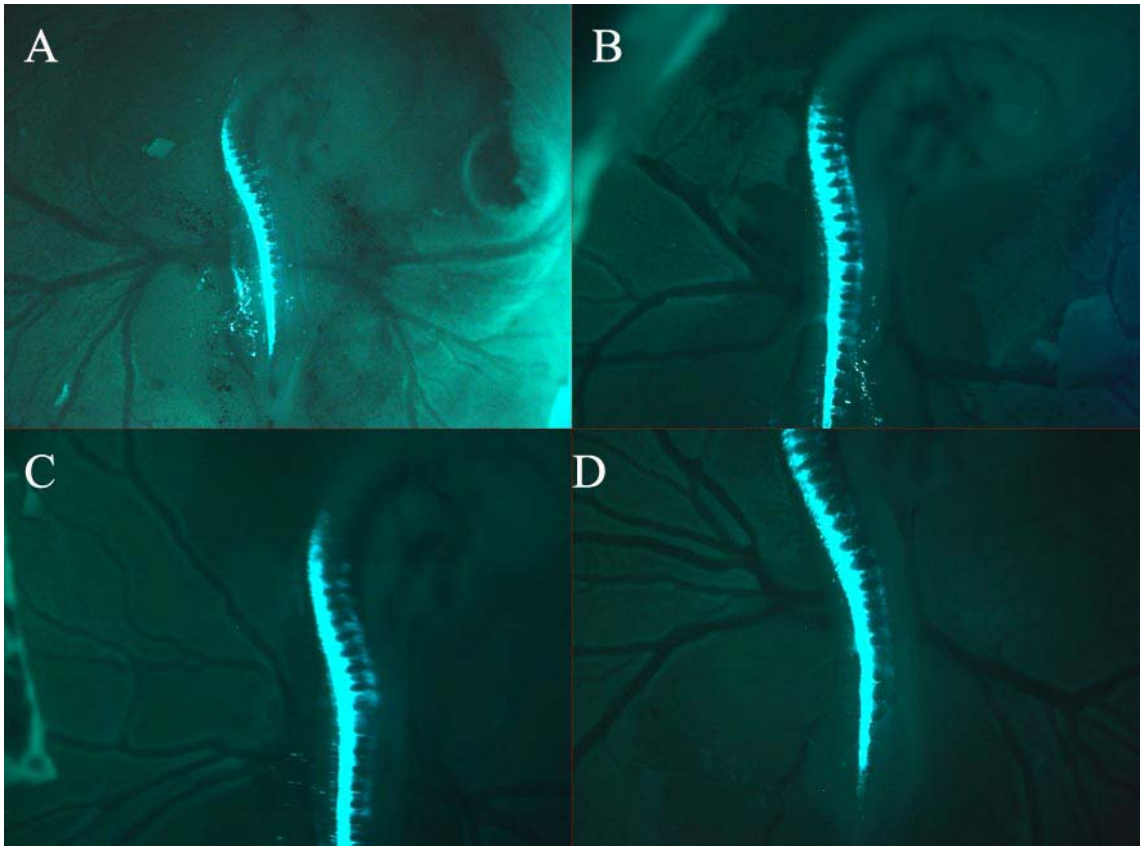


Fig 4.2: The second control of HM electroporation. (A, B, C, D) Images were taken 20-24 hours (T_2=20-24hr) after HM ep. Dorsal views of four embryos that were electroporated with pCAGGS-AFP vector. Strong GFP-signals are present in the neural tube and in neural crest cells migrating through the rostral part of somites (segmentation pattern).

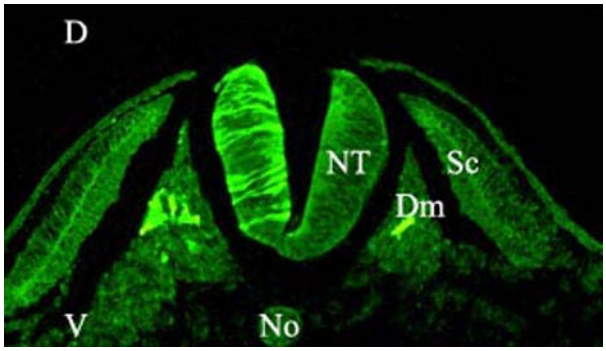
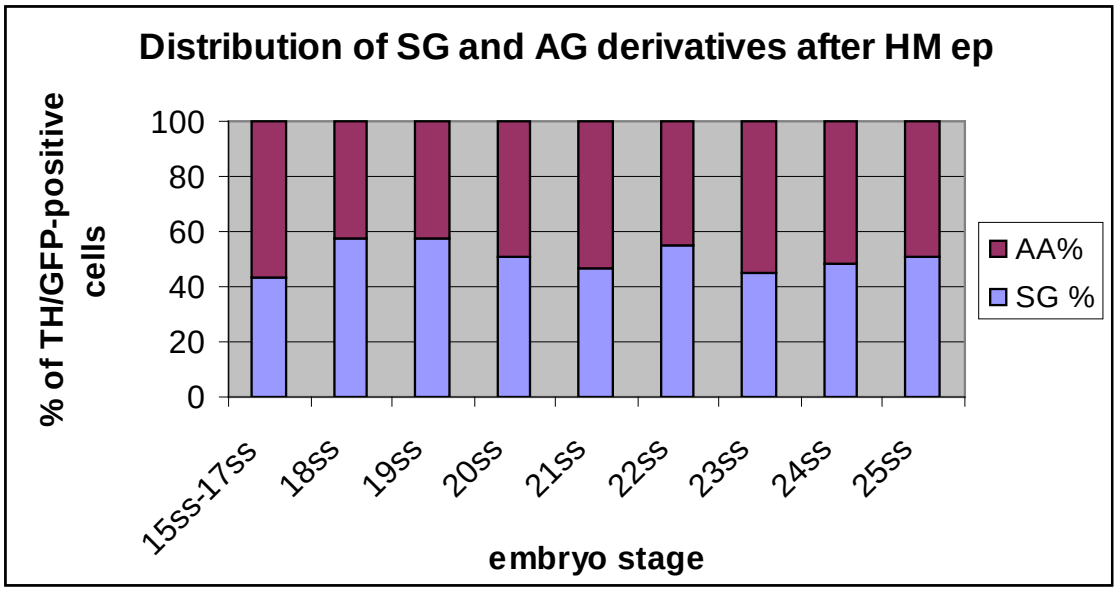


Fig 4.3: Cross section of E 2,5 chicken embryo after HM ep. One half of the neural tube was transfected by GFP hemitube electroporation at embryonic day E2. GFP signals are located in the neural tube and in the migrating neural crest cells. Some labelled NC cells migrated to the contralateral side. D, dorsal; V, ventral; NT, neural tube; Sc, sclerotome; Dm, dermomyotome; No, notochord.

4.1.2 The distribution of NC derivatives after HM ep

Hemitube electroporation (HM ep) was performed in embryos from somite stage 15 till 25 (HH stage 12-15). After HM ep, the numbers of double positive cells for GFP and TH in adrenal anlagen vs. sympathetic ganglia were counted in cross sections of embryos E6.

The distribution of neural crest derivatives in sympathetic ganglia and adrenal gland, respectively, was not different. The proportional distribution of GFP-positive neural crest derivatives after hemitube electroporation was almost equal in both destinations. GFP^+/TH^+ cells in sympathetic ganglia amounted to 50,58% ± 4,17 and in the adrenal glands 49,42% ± 4,16 of all GFP/TH-positive cells in both locations (Graph 4.1).



Graph 4.1: The proportional distribution of neural crest derivatives in sympathetic ganglia (SG) and adrenal glands (AG), respectively. The distribution of GFP/TH-positive neural crest derivatives was almost equal in both destinations, for sympathetic ganglia 50,58% ± 4,17 and for adrenal glands 49,42% ± 4,16.

4.2 Single-cell lineage analysis *in ovo*

To analyse whether a neural crest-derived sympathoadrenal progenitor is capable to produce both types of catecholamine cells, chromaffin cells of adrenal medulla and sympathetic neurons in sympathetic ganglia, respectively, I performed *in ovo* single neural crest cell labelling at the neural tube. Different technological strategies of single-cell labelling were performed in order to find out the most efficient technique.

4.2.1 Ventral-to-Dorsal electroporation

As indicated by the terminology, in this type of electroporation the DNA moves in ventro-dorsal direction. The plasmid solution is injected into the lumen of the neural tube. The electrical field formed between negative and positive electrodes drives the plasmid toward the dorsal aspect of an embryo and solely the NC cells in the dorsal midline of the neural tube are transfected (Figure 4.4).

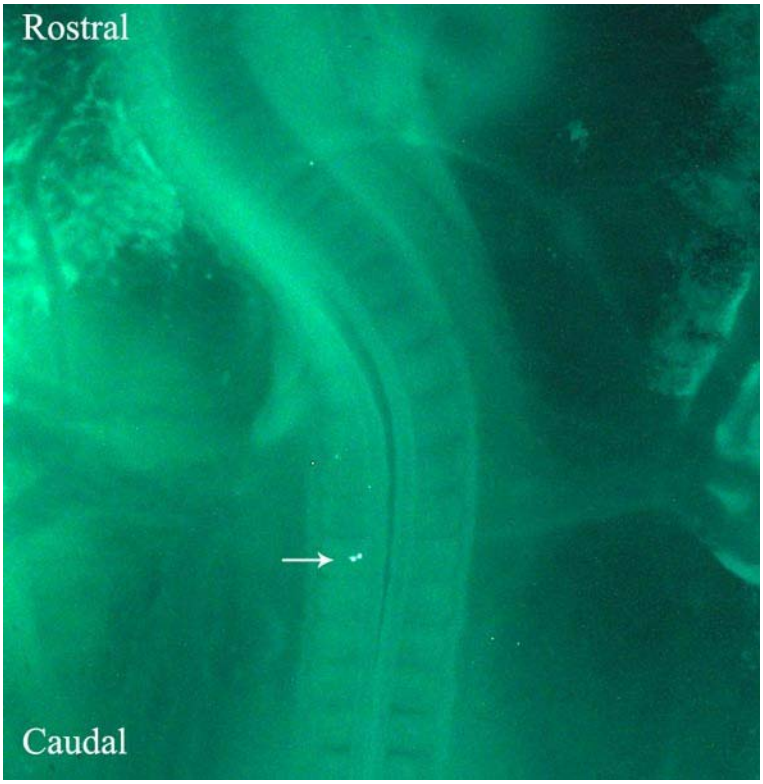


Fig 4.4: Dorsal view of VD-electroporated embryo at T_2=24hr. The labelled NC cells (arrow) have emigrated from the neural tube and move through somite.

The precise positioning of negative and positive electrodes, the size and shape of electrodes, electrical conditions, and plasmid concentrations played the most critical roles in the efficiency of single-cell labelling by ventral-to-dorsal electroporation. In order to define the appropriate protocol for single-cell labelling by VD electroporation, these critical conditions were studied. The vector pCAGGS-AFP was used for VD ep. The concentration of plasmid solution that was injected into the lumen of NT was 1µg/µl.

The efficiency in labelling of a discrete population of cells – from 1 up to 3 cells- by ventral-to-dorsal electroporation reached 5,3%.

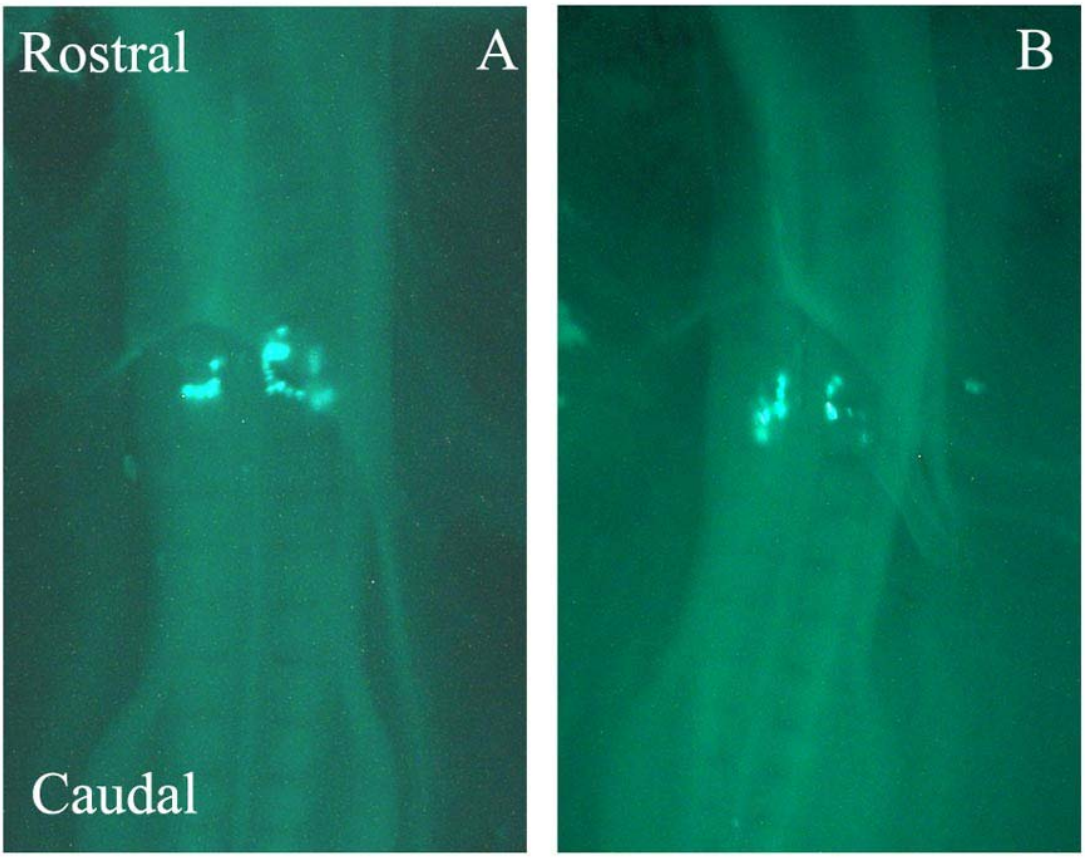


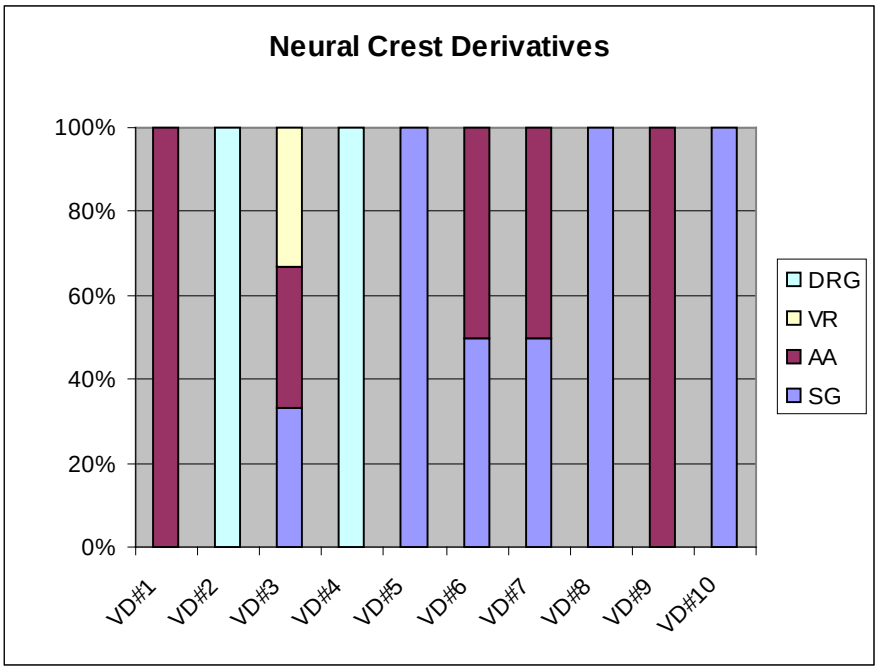
Fig 4.5: Dorsal view of two different VD-electroporated embryos at T_2=24hr. Both (A) and (B) electroporated embryos showed strong GFP signals in delaminating NC cells, there is no residual GFP labelling in the neural tube.

By VD electroporation the neural crest cells in the midline could be transfected. In most of the cases, the VD ep resulted in stronger GFP transfection, and several waves of neural crest delaminating cells were labelled (Figure 4.5). No residual GFP labelling remained in the neural tube at the time of the second control T_2=24hr. This indicates that only groups of cells in the dorsal midline and laterally to the midline were transfected.

4.2.2 The distribution of NC derivatives after VD ep

In the minority of events, in 5,3% of all VD electroporated embryos, this technique came out with the labelling of discrete population (1 - 3 cells) of neural crest cells at the dorsal aspect of the neural tube. In these cases of single-cell labelling, only several types of the NC derivatives were found in the analysed embryos at E6. The analysis was done in cross paraffin sections (10µm) by counting of immunolabelled cells in the final destinations of crest cells. The immunostaining for GFP and TH was performed.

GFP and TH double positive cells were found in sympathetic ganglia and adrenal glands. In two cases of single-cell labelled embryos, the derivatives were located in both destinations, in sympathetic ganglia and adrenal glands, respectively. In three cases, the GFP^+/TH^+ derivatives were found in sympathetic ganglia only. In two cases, GFP+/TH+ cells populated only adrenal glands. In two cases, GFP-positive cells were found in dorsal root ganglia only. One embryo showed three types of derivatives (cells in SG, AA and VR) that originated from the discrete VD ep (Graph 4.2).



Graph 4.2: Distribution of NC derivatives in their final destinations after VD electroporation. DGR, dorsal root ganglia; VR, ventral root; AA, adrenal anlagen; SG, sympathetic ganglia.

Not every embryo that was successfully single-cell electroporated revealed the GFP immunoreactivity at the time of analysis at E 6.

VD ep showed low efficiency in the number of discrete labelling of neural crest progenitors in the midline of the neural tube. This method is apparently not optimal for single-cell labelling to exploit it for proving of our working hypothesis.

4.2.3 Microinjection

As the ventral-to-dorsal electroporation resulted in the labelling of group of cells in most of the trials, we decided to perform the non-electrical method for GFP-labelling. We took advantage of delivering DNA by a pressure injector (Nanoliter 2000, WPI, USA).

This injector was able to incorporate plasmid solution into the cells in the range from nanoliter volumes up to 5µl. In order to calibrate the conditions for single-cell labelling, we injected different plasmid concentrations of 1µg/µl and 2µg/µl. The E2 chicken NC cells were transfected *in ovo* with GFP-construct (pCAGGS-AFP).

4.2.4 The efficiency of microinjection

For microinjection the vector pCAGGS-AFP was used. By this technique, I achieved the single-cell GFP-transfection (Figures 4.6 and 4.7) in 0,3% of all experiments. Unfortunately, none of successfully transfected embryos revealed GFP immunoreactivity at E6. Microinjection did not result in a sufficient number of single-cell injected embryos.

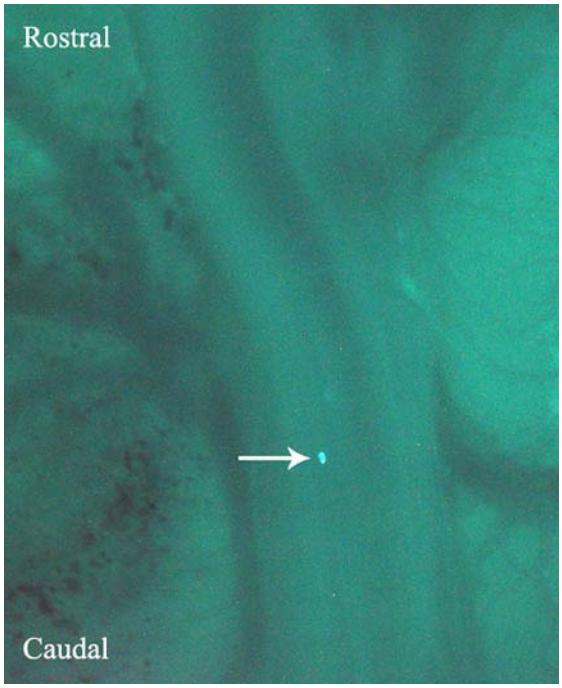


Fig 4.6: Dorsal view of microinjected embryo at T1=3hr.

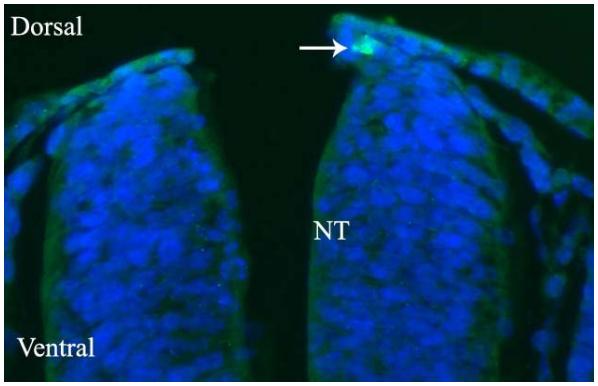


Fig 4.7: Cross section of chicken embryo E2 after microinjection, fixed at T_1=3hr. Single neural crest cell (arrow) is marked by GFP.

4.2.5 Micropipette electroporation

The micropipette electroporation (Mep), which I performed in our lab was developed in collaboration with the Kalcheim laboratory in Jerusalem. The method described by Haas and colleagues served as a basis of this technique (Haas, Sin et al., 2001). I have proved that this method was the best approach for *in ovo* single-cell labelling of the neural crest cells at the dorsal midline of the neural tube among the techniques I have employed.

By the method of micropipette electroporation, the cells that touch the tip of the micropipette are transfected as the electric pulse is applied. The negative electrode is inserted into the micropipette filled with DNA solution and the positive electrode is placed bellow the embryo.

By transfection, the vector pCAGGS-AFP is incorporated into the cytoplasm. As the cell proceeds in proliferation, the plasmid concentration becomes lower. To prevent the dilution of plasmid, so-called “plasmids with stable integration” (pT2K-CAGGS-EGFP, pCAGGS-T2TP) were used (Sato, Kasai et al., 2007). The plasmids of stable integration were co-electroporated at a 1:1 ratio into the single NC cell in the dorsal neural tube. The concentration of plasmid solution was 1 µg/µl.

4.2.6 Efficiency of single-cell labelling by Mep

For the successful single cell electroporation several steps are critical. The best results were achieved at the plasmid concentration of 1 µg/µl, and at the following electrical conditions: 4 pulses of amplitude 50V, 15ms long, interval of 100ms, micropipette resistance of 1,9 -2,0 MΩ.

The efficiency in single-cell electroporated embryos I reached was close to 11% (exactly 10,62%) with a tendency of increasing efficiency according to longer experimental experience. Therefore, the micropipette electroporation seemed to be a suitable technique for single-cell labelling of the NC cells.

4.2.7 Control of single-cell electroporation

After transfection (Mep), the GFP expression was controlled twice *in ovo* under the stereomicroscope equipped with epifluorescence. The first control was done six hours (T_1=6hr) after the electroporation and the second control at 20-24 hours (T_2=20-24hr) after transfection. At T_1, the GFP labelled cell should be located in the midline of the

neural tube; at T_2, the cell and its daughter cells should start to delaminate and no residual GFP expression should remain in the neural tube.

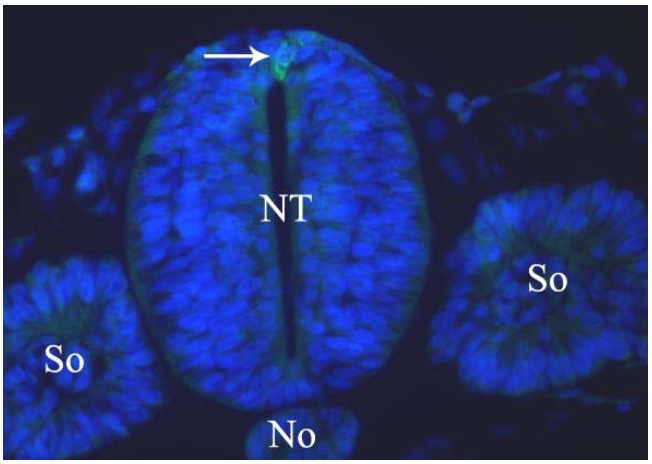


Fig 4.8: Cross section of chicken embryo E2 that was fixed at T_1=6hr after Mep. Nuclear DAPI (blue) staining and GFP immunostaining shows single-labelled cell (arrow) in the dorsal midline. NT,neural tube; No, notochord; So, somite.

The controls at both time points are an important criterion for determining whether one or more cells were labelled. To prove the discrete labelling, a series of embryos was fixed and analysed for GFP-immunoreactivity in cross sections immediately after the first control at T_1 (Figure 4.8 and 4.9 and 4.10).

The transfection that was judged stereomicroscopically *in ovo* at T_1 revealed single cell labelling in 65% (26 embryos) cases (Figure 4.9) and double-cell labelling in 35% (14 embryos) cases (Figure 4.10).

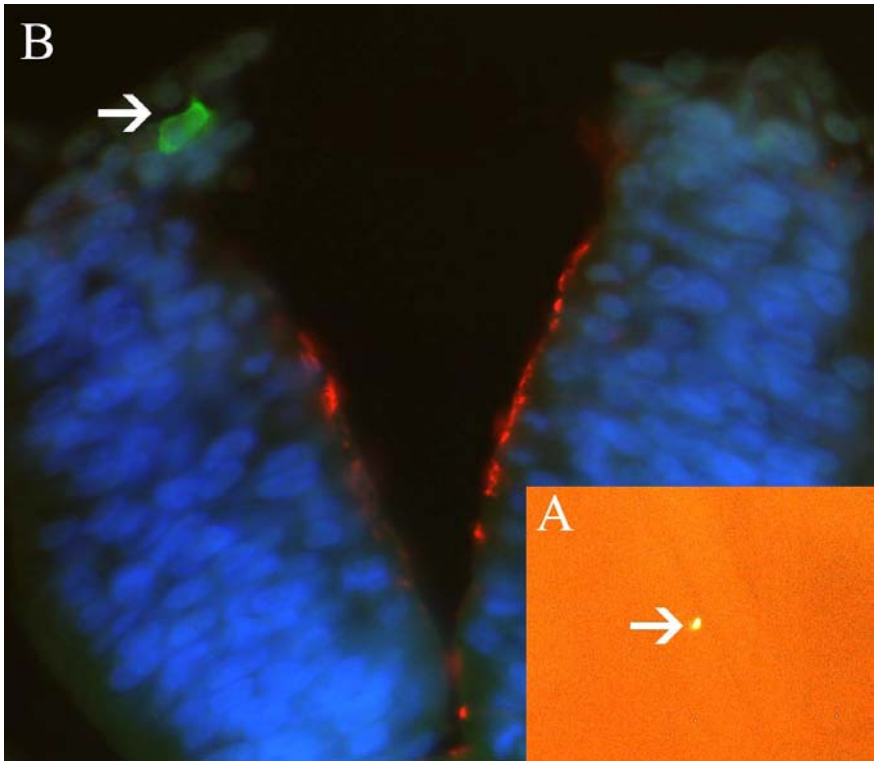


Fig 4.9: The first control of single-cell electroporation at T_1= 6hr. (A) Dorsal view of single-cell (arrow) electroporated chicken embryo (Mep technique). Two plasmids (plasmids of stable integration) were co-transfected: pCAGGS-T2TP and pT2K-CAGGS-EGFP. (B) The cross section of the same embryo as depicted in (A). The neural tube with a GFP-labelled single-cell (arrow) in the dorsal midline. The neural crest cell shows GFP (green) immunoreactivity. The labelled cell is not positive for HNK1 (red) immunoreactivity yet. The NC cells start to express the HNK1 epitope after they detach from the neural tube. DAPI (blue) stains nuclei.

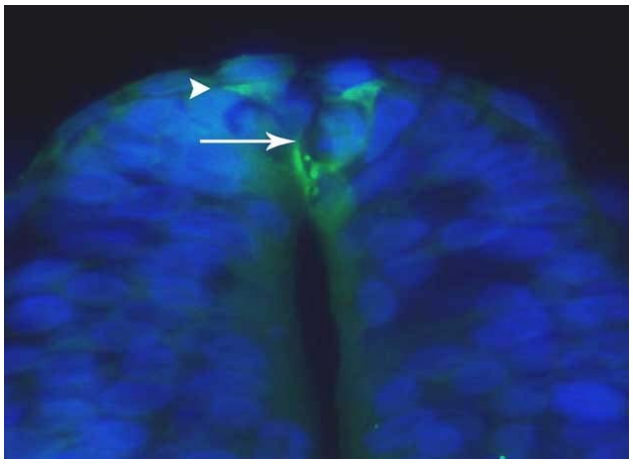


Fig 4.10: Transverse section of a Mep transfected embryo, where two individual cells were labelled. A GFP-positive-cell in the focus (arrow) is labelled in the dorsal midline of the neural tube. The second neural crest cell (arrowhead) is not in focus, i.e. only part of the cell shows GFP immuoreactivity.

4.2.8 Distribution of NC derivatives after single-cell electroporation

After single cell electroporation, the embryos were kept in the incubator till E6 when adrenal gland and sympathetic ganglia are developed. Embryos were fixed at E6 and processed for immunohistological analysis. The distribution of GFP/TH-positive cells within their final destinations was then analysed. Both sympathetic neurons and chromaffin cells share catecholaminergic properties.

In 14 cases (87,5%) of single cell GFP-transfected embryos, we have found a dual progeny located in both sympathoadrenal destinations, the sympathetic ganglia (Figure 4.12) and the adrenal glands (Figure 4.13). Numbers of labelled derivatives were always lower than twenty cells in sympathetic ganglia or adrenal glands per embryo.

Derivatives of single-labelled cells were found in the location of adrenal gland at the level of electroporation. In contrast, the derivatives located in the sympathetic ganglia were found rostrally to the level of electroporation in each analysed embryo. The distance between the derivatives in sympathetic ganglia and adrenal glands along the rostro-caudal axis was two to three segments (Figure 4.11).

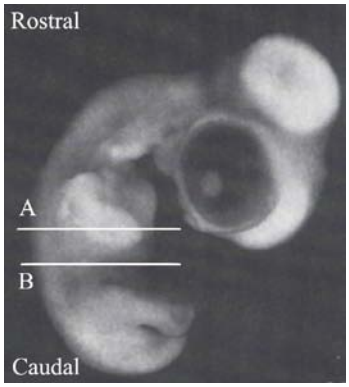


Fig 4.11: Location of the NC derivatives along the rostro-caudal axis, A-line depicts the level, where sympathetic ganglia were populated, B-line shows the level of electroporation, where the adrenal gland was populated.

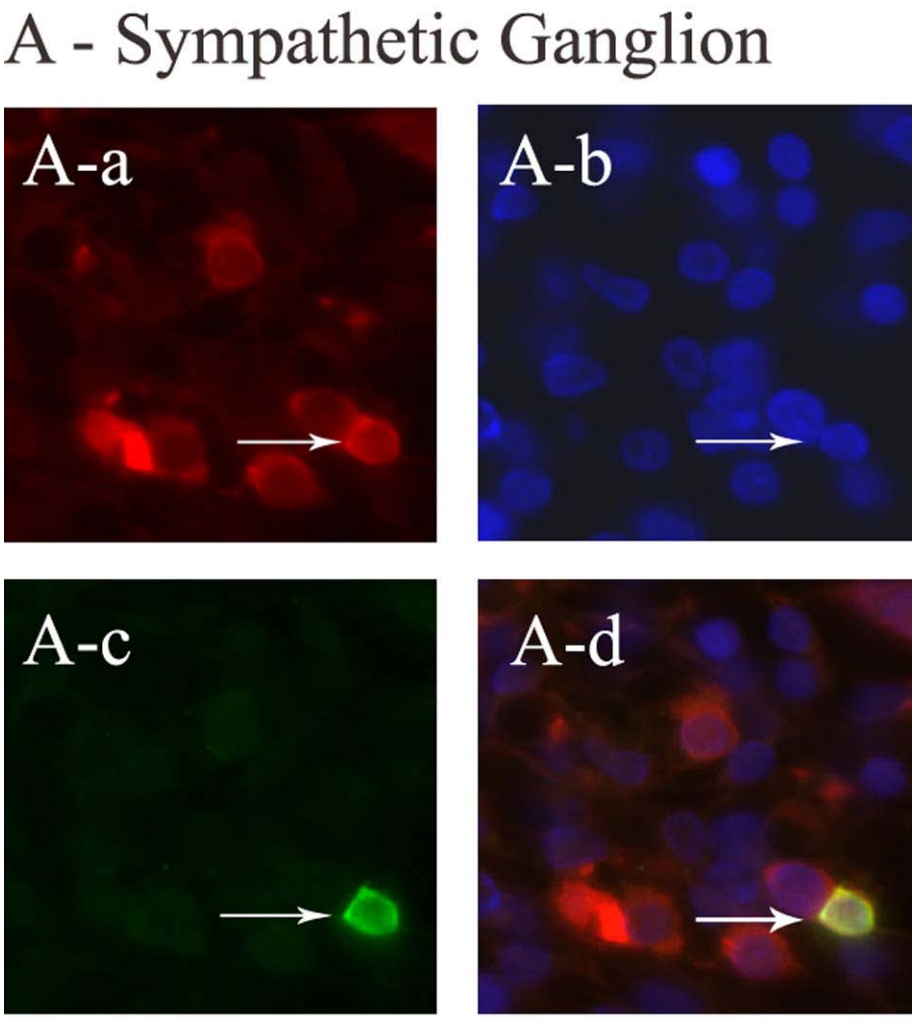


Fig 4.12: Derivatives of a single GFP-labelled neural crest cell were found in sympathetic ganglia. Area depicts one labelled cell (arrow), **(B-a)** TH immunoreactivity, **(B-b)** nuclear staining DAPI, **(B-c)** GFP immunoreactivity, **(B-d)** merge.

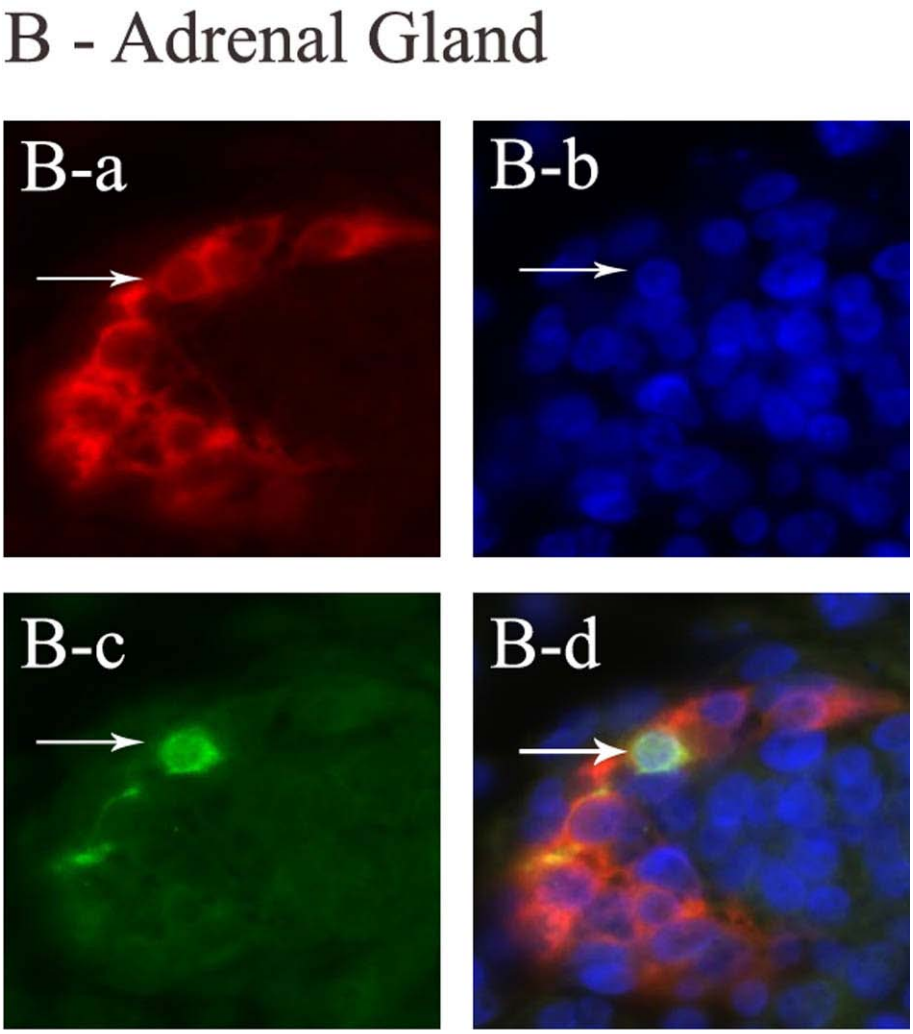


Fig 4.13: Derivatives of a single GFP-labelled neural crest cell were found in the adrenal gland. Area depicts one labelled cell (arrow), **(B-a)** TH immunoreactivity, **(B-b)** nuclear staining DAPI, **(B-c)** GFP immunoreactivity, **(B-d)** merge.

Single-cell labelling of neural crest cells at the level of the neural tube resulted not only in the derivatives located in both the adrenal gland and sympathetic ganglia. One embryo that was electroporated at the level opposite to the 23rd somite displayed only one type of NC derivative. The GFP-labelled cells were found in the adrenal gland. Another two embryos that were electroporated opposite to the level of the 19th somite showed derivatives located in the sympathetic ganglia.

Our single-cell lineage analysis was focused on sympathoadrenal derivatives. However, several single-cell electroporation trials gave rise to derivatives located only in DRG (16 embryos), only in ventral root (5 embryos) and derivatives located in DRG plus ventral root (11 embryos).

4.3 Chicken sympathetic ganglia contain chromaffin cells

Recently, the presence of chromaffin-like cells in E8 chicken sympathetic ganglia was described in a study dealing with the effects of persistent BMP4-signals on the secondary sympathetic ganglia at the upper thoracic level. NF-M *in situ* hybridization analysis in combination with TH immunohistochemistry revealed about 25% of chromaffin-like cells in avian sympathetic ganglia. Chromaffin-like cells were positively labelled by TH-immunoreactivity and did not express mRNA for NF-M (Huber, Franke et al., 2008). This observation raises the possibility that GFP-positive/TH-positive cells found in our single-cell NC ep´s within sympathetic ganglia could in fact be chromaffin cells rather than neurons. To approach this issue, I started to do double-labelling for NF mRNA and TH.

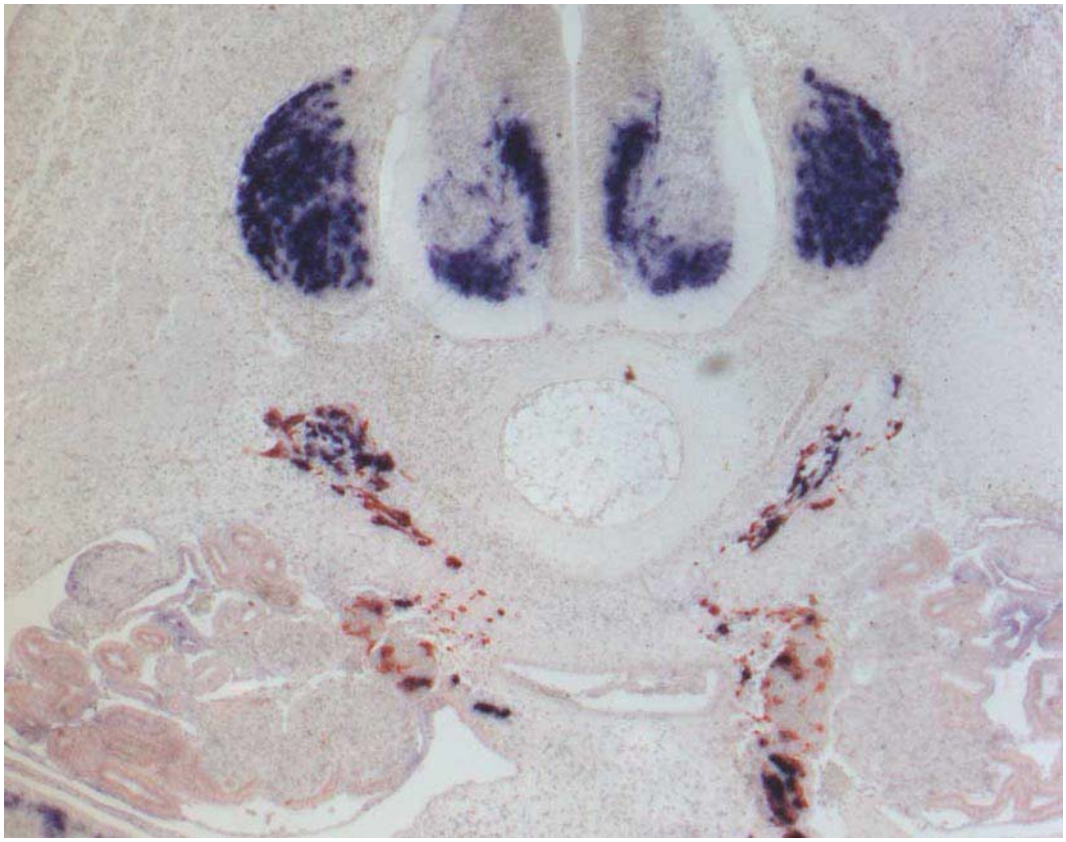


Fig 4.14: Cross section of a E6 chicken embryo at the adrenal gland level, *in situ* hybridisation for NF-M (dark blue) and immunohistochemistry for TH (red). In sympathetic ganglia and adrenal glands cells positive for TH immunoreactivity are located. mRNA for neurofilament-M is expressed in NT, DRG, SG, and AG.

Four chicken embryos of embryonic day E6 were analysed. The numbers of $TH^+/NF\text{-}M^+$ and $TH^+/NF\text{-}M^-$ cells were counted in every fifth cross section. The analysed area started at the rostral end of the adrenal gland and continued 140µm caudally.

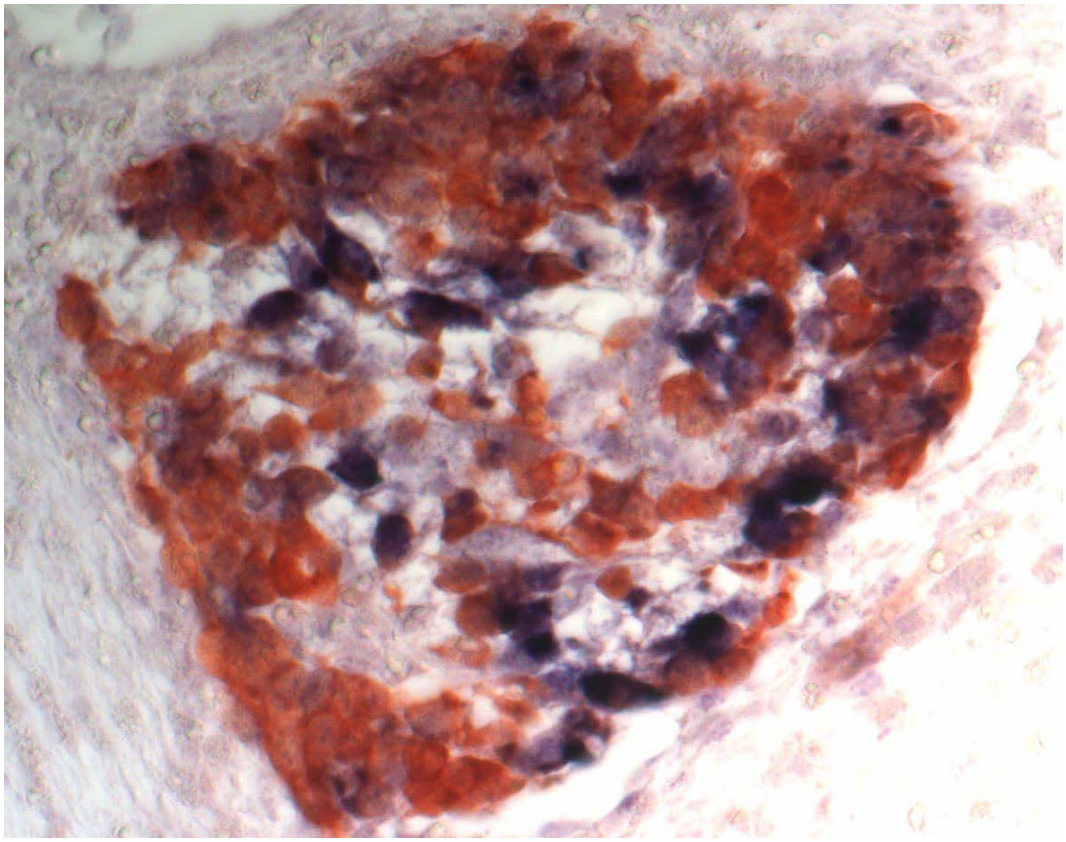
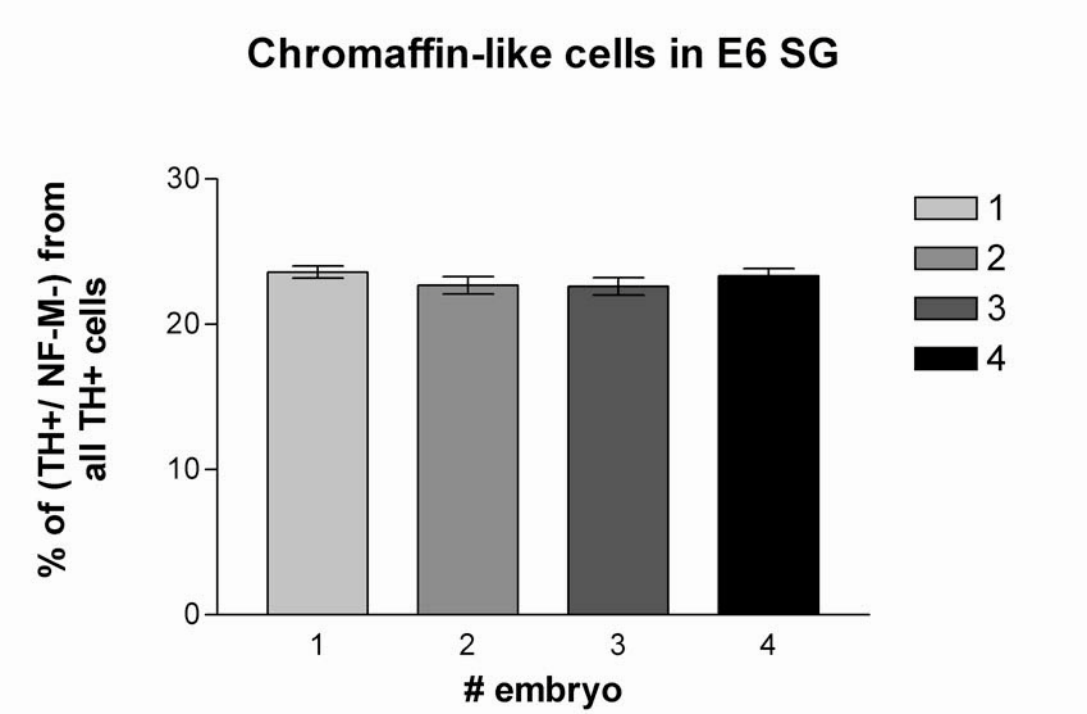


Fig 4.15: Sympathetic ganglion of a E6 chicken embryo; ISH for NF-M (dark blue), IHC for TH (red); taken with 40x objective.

Our analysis revealed TH-positive and NF-M-negative chromaffin (chromaffin-like) cells in the examined areas (Figure 4.14). The portion of chromaffin-like cells in sympathetic ganglia represented about 23,06% ± 2,35 of the total number of ganglionic TH-positive cells. The TH-positive/NF-M-positive cells of chicken sympathetic ganglia of E6 embryos represent autonomic neurons. The majority of chromaffin-like cells shown as TH-positive/NF-M-negative are located in the periphery of the sympathetic ganglia (Figure 4.15).

The statistical analysis has not showed any significant differences in the numbers of chromaffin-like cells within SG among the analysed group of four embryos (Graph 4.3).



Graph 4.3: Chromaffin-like cells are found in E6 chicken SG. Numbers of chromaffin-like cells ($TH^+/NF\text{-}M^-$) are depicted as a percentage of tyrosine hydroxylase positive cells in SG. Data are presented as means ± standard error of the mean (SEM).

It has been described previously that NF-M-positive cells are present in the area of avian adrenal anlagen. Cells located preferentially at dorsal and ventral aspect of the dorsal aorta express NF-M mRNA at embryonic day 5 (Ernsberger, Esposito et al., 2005). In our *in situ* hybridisation analysis we have found NF-M dark blue positive cells in the adrenal anlagen at embryonic day 6 (Figure 4.16). The location of NF-M-positive cells confirms the results described previously by Ernsberger and colleagues.

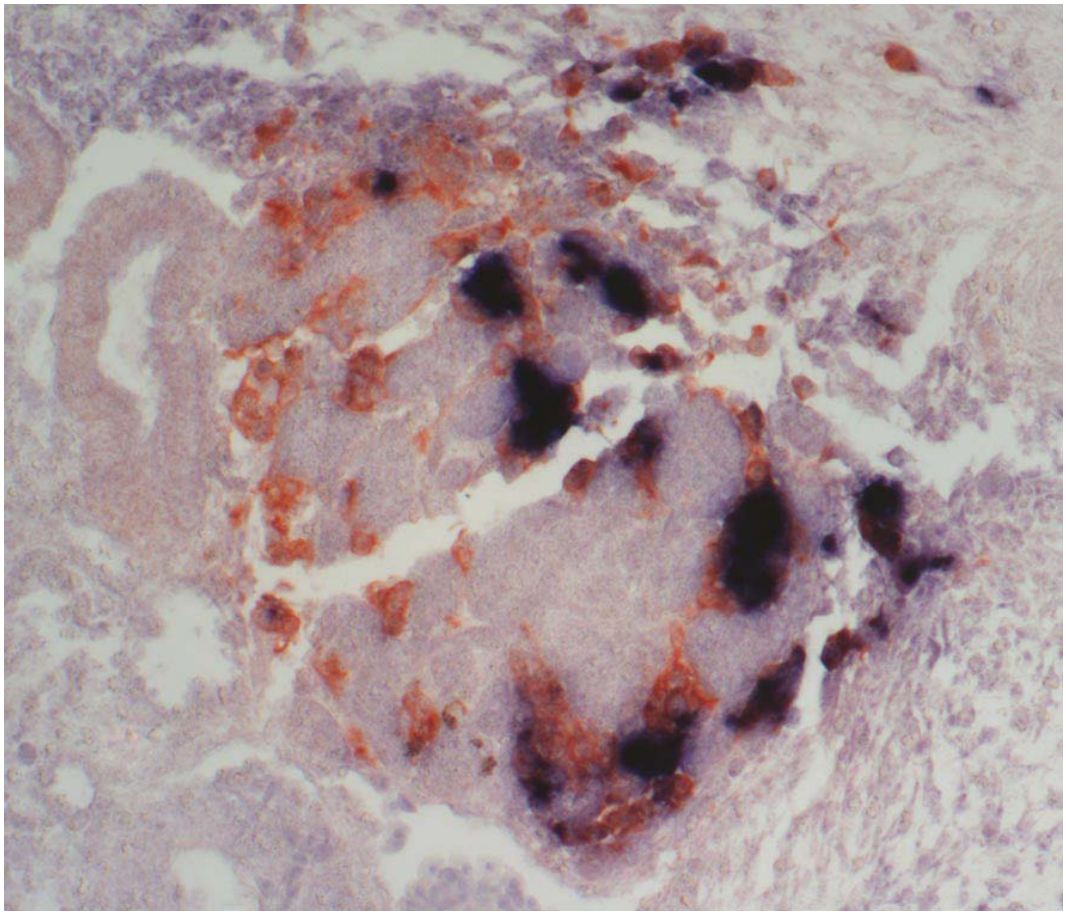


Fig 4.16: Adrenal gland of E6 chicken embryo, i*n situ* hybridisation for NF-M (dark blue) and immunohistochemistry for TH (red).

4.4 Single NC cell gives rise to both sympathetic neurons and chromaffin cells

By micropipette electroporation, we have found that in the great majority of cases, a single-labelled NC cell gives rise to cells located in both sympathetic ganglia and adrenal gland. We have analysed the derivatives of single-labelled cell by TH and GFP immunoreactivity. We have also shown that chicken sympathetic ganglia of E6 embryos contain a portion of chromaffin-like cells. The portion of chromaffin-like cells comprises about 23% of all TH-positive cells within the ganglion. In order to distinguish between sympathetic neurons and chromaffin-like cells within the sympathetic ganglion we performed the immunohistochemistry analysis (anti-TH, anti-GFP) in combination with *in situ* hybridisation analysis for NF-M.

This analysis has revealed that GFP-positive cells located in sympathetic ganglia are sympathetic neurons. In seven cases of single-cell electroporation, we have found $GFP^+/TH^+/NF\text{-}M^+$ cells in sympathetic ganglia (Figure 4.17) and $GFP^+/TH^+/NF\text{-}M^-$ cells

in adrenal glands (Figure 4.18). These results strongly support the hypothesis of common progenitor cell for both sympathetic neurons and chromaffin cells.

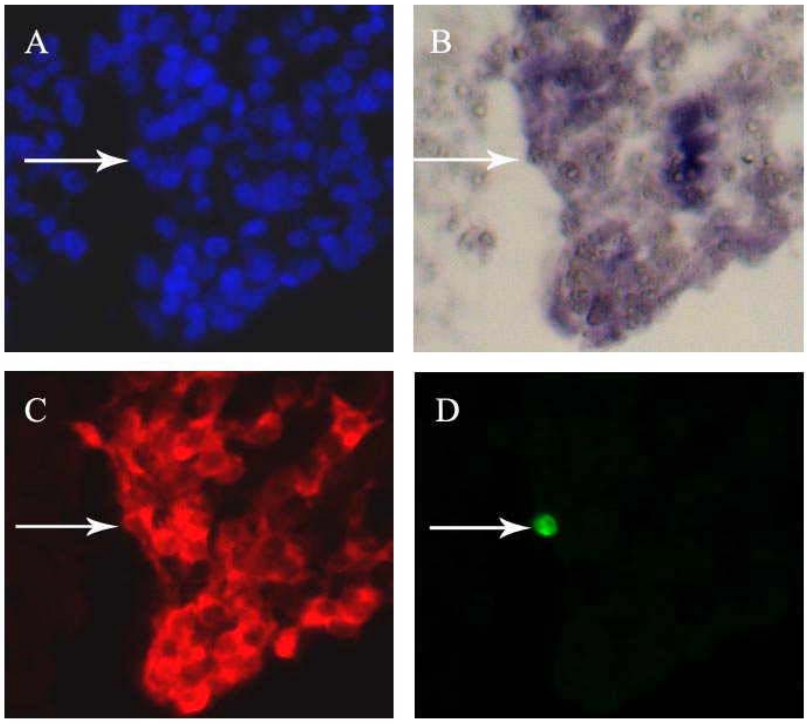


Fig 4.17: $GFP^{+}/TH^{+}/NF\text{-}M^{+}$ cell in sympathetic ganglion (SG) represents a sympathetic neuron. (A) DAPI, nuclear-staining, (B) NF-M mRNA, (C) Immunohistochemistry for TH, (D) IHC for GFP.

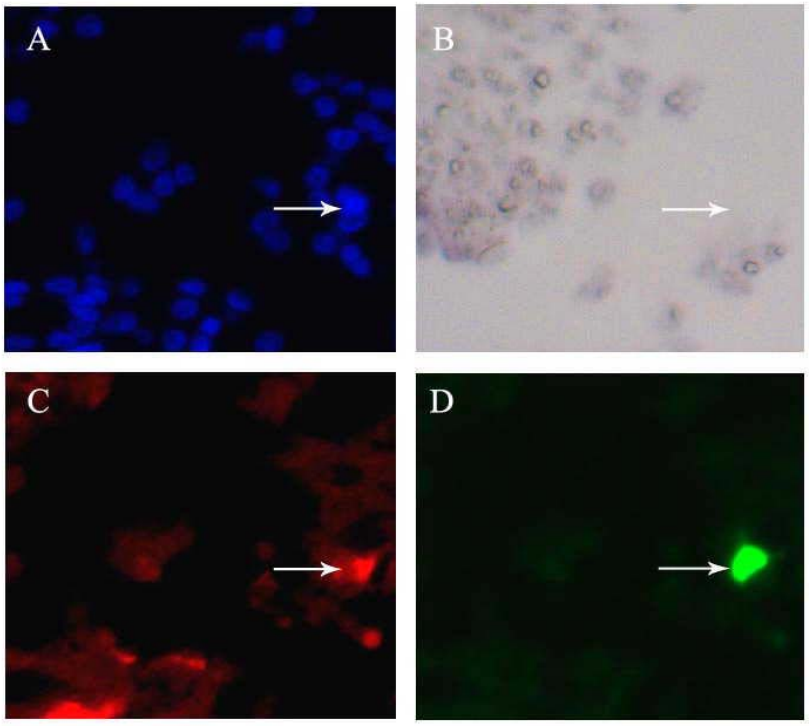


Fig 4.18: $GFP^{+}/TH^{+}/NF\text{-}M^{-}$ cell in adrenal gland (AG) represents a chromaffin cell. (A) DAPI-nuclear-staining, (B) NF-M mRNA, (C) Immunohistochemistry for TH, (D) IHC for GFP.

4.5 Notch signalling

Notch signalling has been shown to be involved in the formation of sympathetic ganglia and dorsal root ganglia (Tsarovina, Schellenberger et al., 2008). We were interested whether to begin to address the issue whether Notch signalling is involved in the phenotypic determination of sympathetic neurons and adrenal medullary cells we performed *in situ* hybridisation analysis for several members of the Notch/Delta pathway. The expression patterns of Delta-like 1 (Dll1), Delta-like 4 (Dll4), Hairy 1, and Hairy 2 have been analysed in chicken embryos of different developmental stages.

Our *in situ* hybridisation analysis has revealed a strong expression of Delta-like 1 mRNA in dermomyotome, neural tube, dorsal aorta and between dorsal aorta and nephric duct at E3 (Figure 4.19). At this stage, the sympathoadrenal cells start to populate the vicinity of the dorsal aorta and the adrenal anlagen starts to be formed in the area between the dorsal aorta and mesonephros. The expression of Dll1-mRNA is lost in the area of adrenal gland at E6 (Figure 4.21). Its expression persists in the neural tube and the dorsal aorta; and is additionally located in the dorsal root ganglia (Figure 4.21).

The analysis of mRNA for Delta-like 4 has shown its expression in the neural tube, the wall of dorsal aorta and in the prospective area of adrenal gland at E4 (Figure 4.22).

The mRNA for Hairy 1 is found in the area between the dorsal aorta and mesonephros at E4 (Figure 4.24); similar to the Dll1 (Figure 4.19) and Dll4 (Figure 4.22) expression pattern. Hairy 1 mRNA persists in this location till E4 (Figure 4.24) and further till E6 (Figure 4.26).

Signals for Hairy 2 mRNA are found in the neural tube and in close proximity of the nephric duct laterally to the dorsal aorta at E4 (Figure 4.27). More extended expression pattern of Hairy 2 could be seen at E6. At this developmental stage, the mRNA for Hairy 2 was present in the area of the neural tube, the dorsal root ganglia, the sympathetic chain and the adrenal anlagen (Figure4.29).

The expression pattern of both Hairy 1 (Figure 4.26) and Hairy 2 (Figure 4.29) at E6 suggests the localization of their mRNAs in the sympathoadrenal cells in the adrenal glands and primary sympathetic ganglia.

However, the in situ hybridisation for Notch signalling members and the anatomical localisation does not prove the identity of mRNA-positive cell. The co-labelling with NC-specific or catecholaminergic markers has to be performed in order to determine the involvement of Notch signalling in the development of sympathoadrenal lineage.

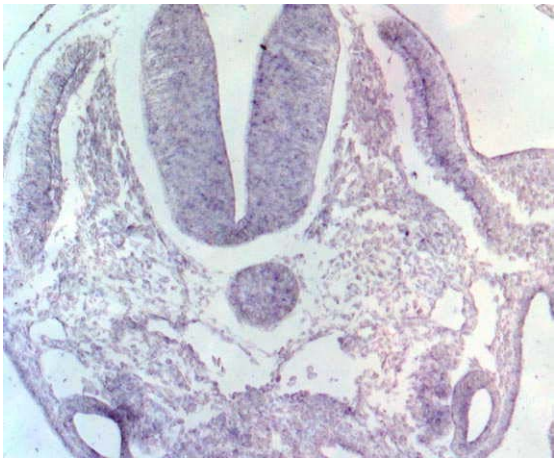


Fig 4.19: The expression pattern of Delta-like 1 (Dll 1) mRNA in E3 chicken embryo at the trunk level. The Dll1 mRNA is expressed in neural tube, dermomyotome, dorsal aorta, and between dorsal aorta and nephric duct.

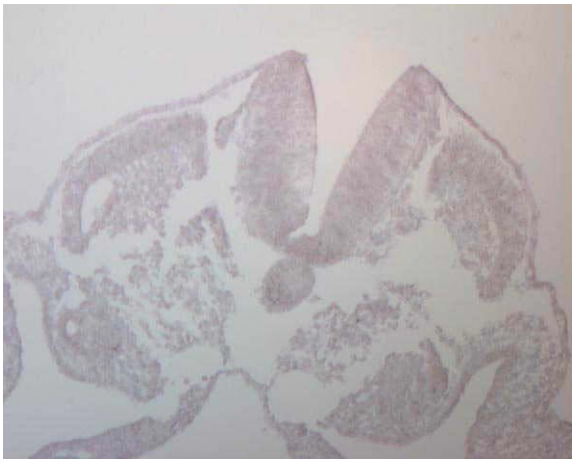


Fig 4.20: Negative control for Dll 1 mRNA expression in the trunk of E 3 chicken embryo. *In situ* hybridisation with sense probe.

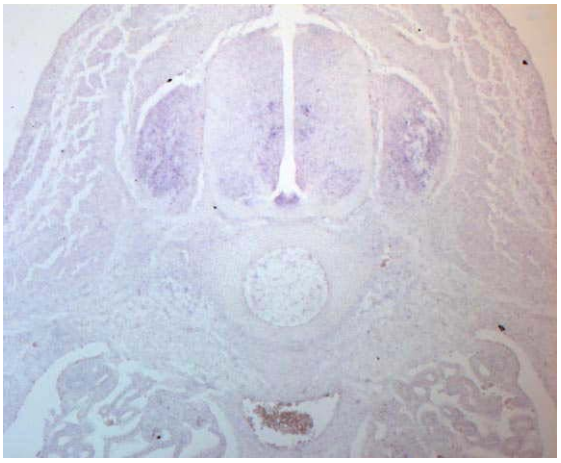


Fig 4.21: The expression pattern of Delta-like 1 (Dll 1) mRNA in E6 chicken embryo at the trunk level. The Dll1 mRNA is expressed in neural tube, dorsal root ganglia, and week expression in dorsal aorta.

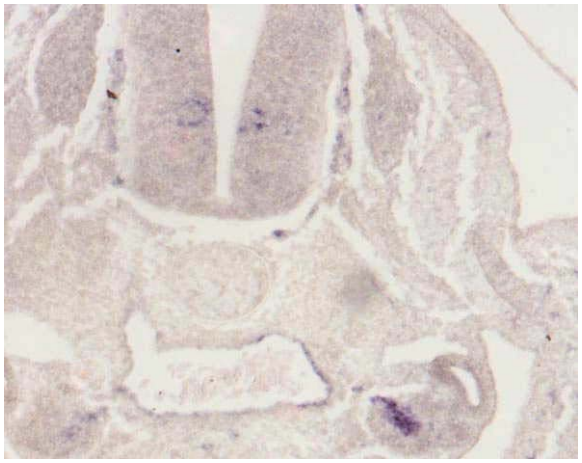


Fig 4.22: The expression pattern of Delta-like 4 (Dll 4) mRNA in E4 chicken embryo at the trunk level. The Dll4 mRNA is expressed in neural tube, dorsal aorta, and between dorsal aorta and nephric duct.

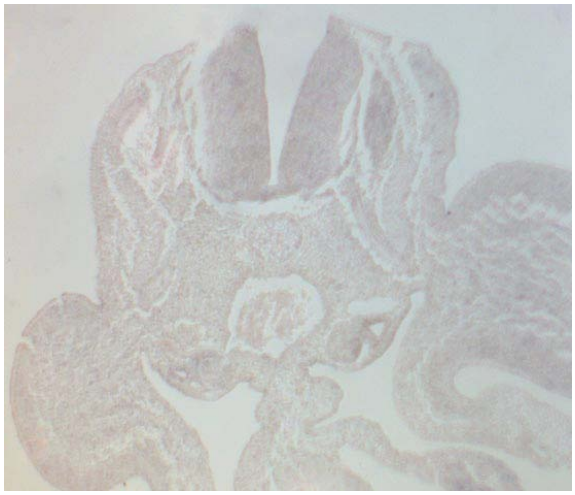


Fig 4.23: Negative control for Dll 4 mRNA expression in the trunk of E 4 chicken embryo. In situ hybridisation with sense probe.

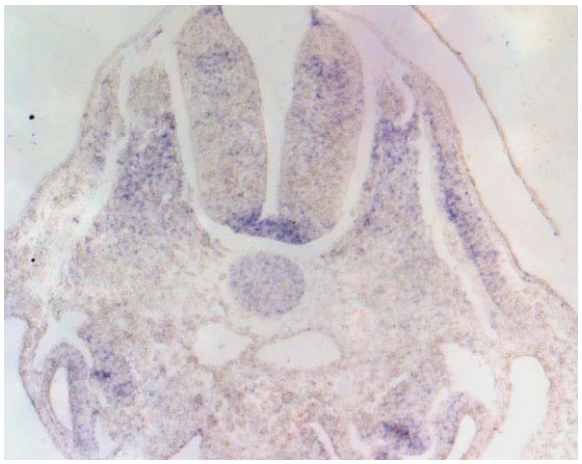


Fig 4.24: The expression pattern of Hairy 1 mRNA in E4 chicken embryo at the trunk level. The Hairy 1 mRNA is expressed in neural tube, dermomyotome, prospective dorsal root ganglia, and between dorsal aorta and nephric duct.

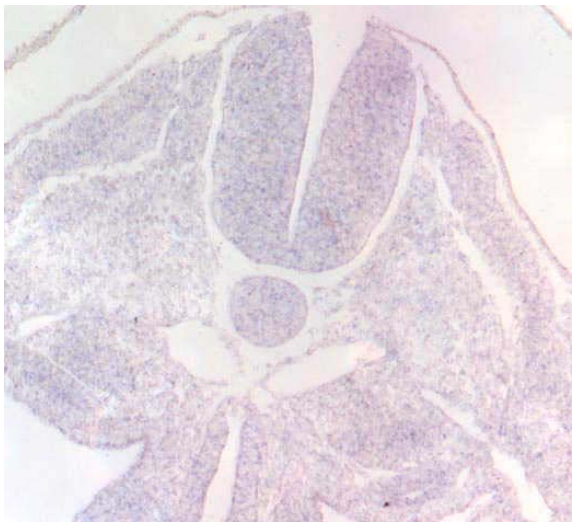


Fig 4.25: Negative control for Hairy 1 mRNA expression in the trunk of E 4 chicken embryo. In situ hybridisation with sense probe.

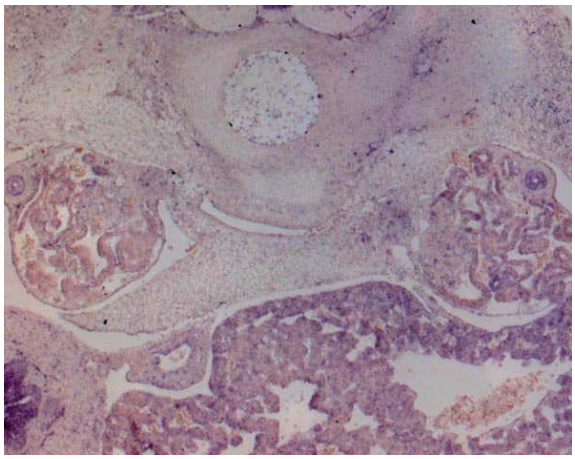


Fig 4.26: The expression pattern of Hairy 1 mRNA in E6 chicken embryo at the trunk level. The Hairy 1 mRNA is expressed in sympathetic ganglia primordium, and between dorsal aorta and nephric duct, where the adrenal gland is formed at E 6.

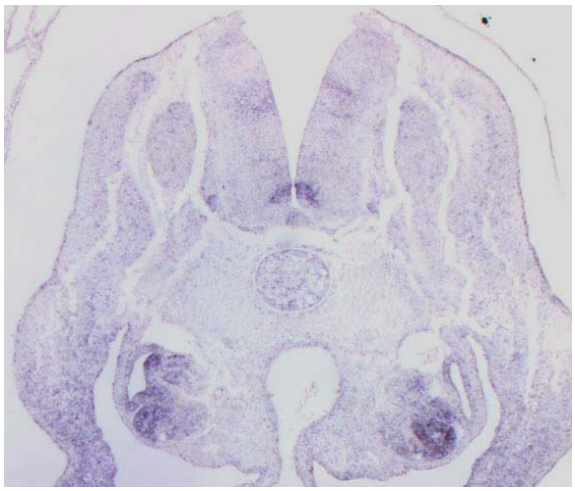


Fig 4.27: The expression pattern of Hairy 2 mRNA in E4 chicken embryo at the trunk level. The Hairy 2 mRNA is expressed in neural tube, and between dorsal aorta and nephric duct.

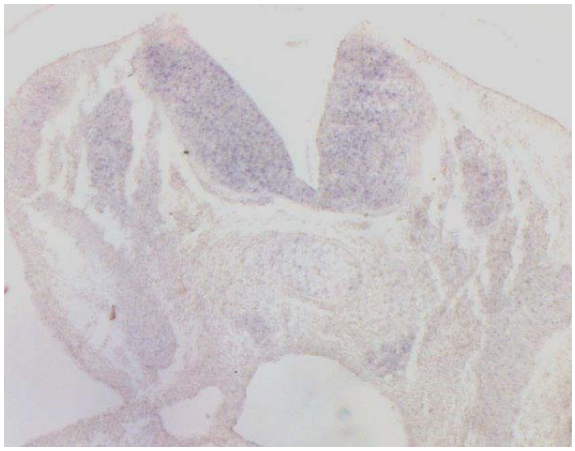


Fig 4.28: Negative control for Hairy 2 mRNA expression in the trunk of E 4 chicken embryo. In situ hybridisation with sense probe.

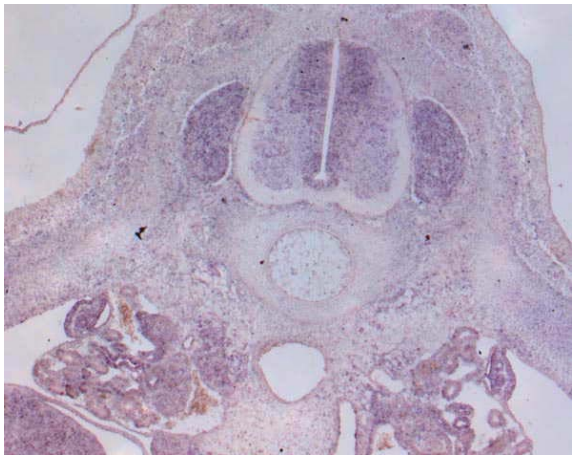


Fig 4.29: The expression pattern of Hairy 2 mRNA in E6 chicken embryo at the trunk level. The Hairy 2 mRNA is expressed in neural tube, dorsal root ganglia, sympathetic ganglia primordium, and between dorsal aorta and nephric duct.

4.6 Vascular pattern of adrenal gland and sympathetic ganglia

The adrenal gland, in contrast to sympathetic ganglia, is an endocrine organ which requires an intense blood supply. We were interested whether and how the vascular pattern of the developing adrenal gland differs from developing sympathetic ganglia. We took an advantage of the quail model for our analysis of the vascular pattern. The specific quail marker QH1 stains for endothelial cells. We have combined immunohistochemistry for QH1 with HNK1 staining, which labels migrating neural crest cells.

Our analysis has shown that NC cells (HNK-1^{+}) migrate to the proximity of the dorsal aorta already at E2-E2,5 in quails (Figures 4.30 and 4.31). The NC cells are beginning

to invade the area dorsally and laterally to the dorsal aorta starting on E2,5 onward (Figure 4.31 and 4.32). At E3,5, the HNK-1$^+$ cells were present laterally to the dorsal aorta and started to form primary sympathetic chain and adrenal anlagen (Figure 4.33). The endothelial cells (QH1$^+$) were present in the prospective areas of sympathetic chain and adrenal anlagen at late E3 and E3,5 (Figures 4.32 and 4.33). At E3,5, more endothelial cells were found in the area of adrenal glands than of primary sympathetic ganglia (Figure 4.33). The vascular pattern has shown differences in the target regions populated by sympathoadrenal cells at E4-E4,5 (Figures 4.34 and 4.35). The QH1$^+$ cells that populated the primary sympathetic chain were found in the great majority on the margins of the forming ganglia. Only scarce endothelial cells were located in the ganglia (Figures 4.34 and 4.35). In contrast to the QH1$^+$ cells which settle in the area of sympathetic ganglia, the endothelial cells of adrenal anlagen were predominantly present within the gland and on its margins (Figures 4.34 and 4.35). More prominent differences in the vascular pattern between adrenal glands and sympathetic ganglia are found at E5 (Figure 4.36).

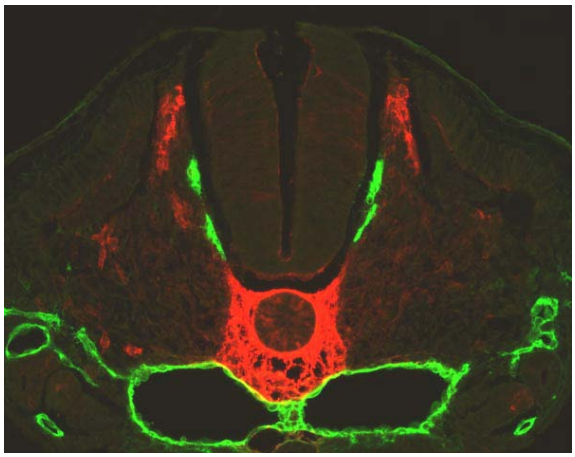


Fig 4.30: Cross section of the trunk of E 2 quail embryo. Immunohistochemistry for QH1 (green) and for HNK1 (red). Few HNK1-positve cells located dorsally to the dorsal aorta. Note, no QH1/HNK1-doublepositive cells are present.

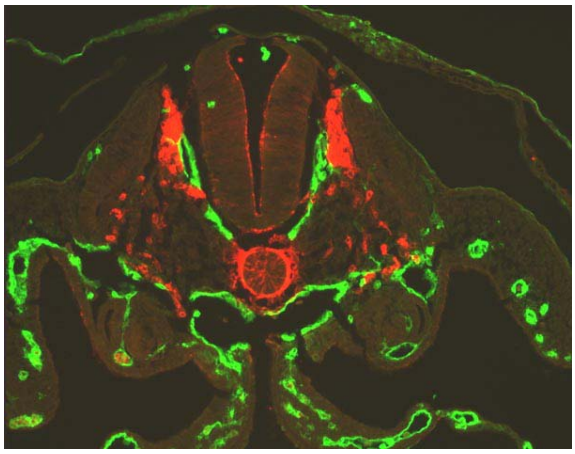


Fig 4.31: Cross section of the trunk of E 2,5 quail embryo. Immunohistochemistry for QH1 (green) and for HNK1 (red). The HNK1-positve cells populate the area dorsally and laterally to the dorsal aorta.

Fig 4.32: Cross section of the trunk of E 3 quail embryo. Immunohistochemistry for QH1 (green) and for HNK1 (red).

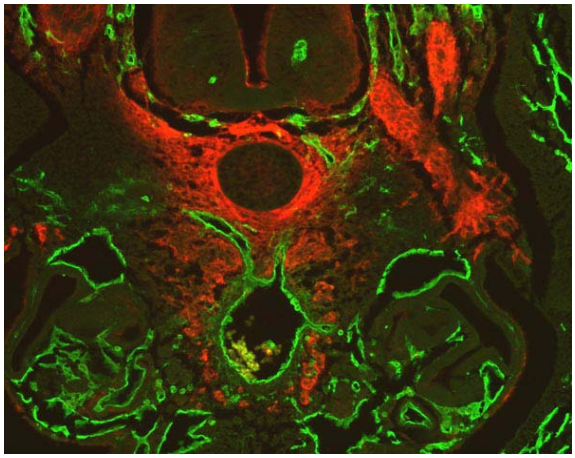


Fig 4.33: Cross section of the trunk of E 3,5 quail embryo. Immunohistochemistry for QH1 (green) and for HNK1 (red). The HNK1-positve neural crest cells of sympathoadrenal lineage invaded the area laterally and ventrally to the dorsal aorta.

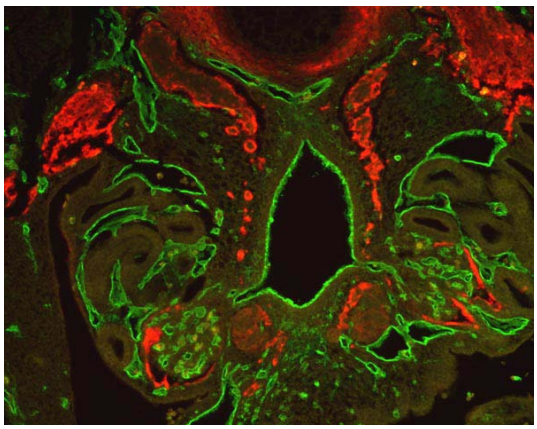


Fig 4.34: Cross section of the trunk of E 4 quail embryo. Immunohistochemistry for QH1 (green) and for HNK1 (red). The HNK1-positve neural crest cells invaded the adrenal anlagen and primary sympathetic chain.

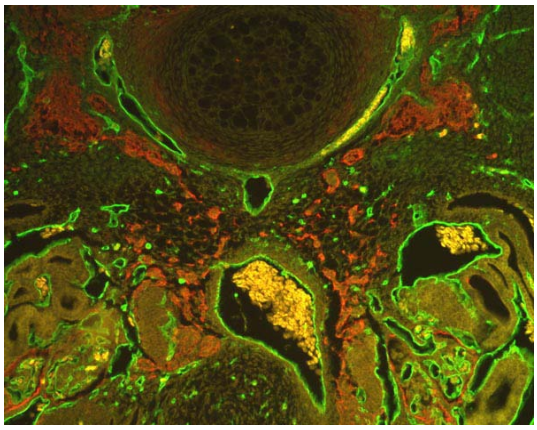


Fig 4.35: Cross section of the trunk of E 4,5 quail embryo. Immunohistochemistry for QH1 (green) and for HNK1 (red). The HNK1-positve neural crest cells populate the adrenal glands and primary sympathetic ganglia. The adrenal glands contain more abundantly endothelial cells in comparison to the primary sympathetic ganglia.

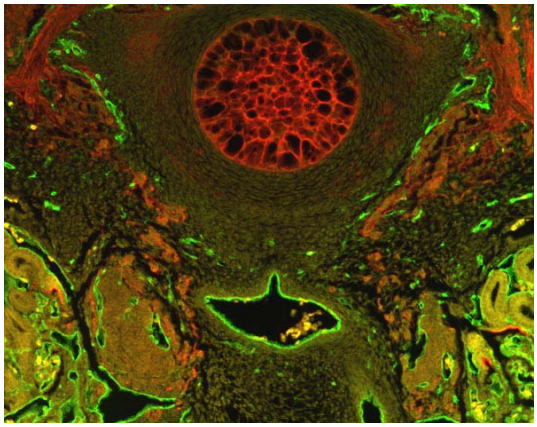


Fig 4.36: Cross section of the trunk of E 5 quail embryo. Immunohistochemistry for QH1 (green) and for HNK1 (red). The HNK1-positve neural crest cells populate the adrenal glands and primary sympathetic ganglia. The adrenal glands show endothelial cells within the organ and on its margins. The sympathetic ganglia contain scarce endothelial cells, which in the great majority are located on the margins of the ganglia.

5 CONCLUSION

We have shown that micropipette electroporation of plasmids stably transfected and expressed in the genome of a single neural crest cell may be used as a method for tracing the sympathoadrenal cell lineage *in ovo.* Our results support the classical hypothesis that sympathoadrenal progenitors represent a common pool for both sympathetic neurons and chromaffin cells.
Furthermore, we have confirmed that chicken sympathetic ganglia contain about one quarter of chromaffin-like cells at E6.

Based on the preliminary data of mRNA expression of Notch/Delta signalling members, we suggest a putative role of Notch signalling in sympathoadrenal development. However, detailed experiments are needed. The preliminary analysis of vascular pattern in the area of developing sympathetic ganglia and adrenal glands has revealed the differences in the densities of endothelial cells possibly suggesting differences in vascular densities. The analysis of molecular differences between endothelium of sympathetic ganglia and adrenal glands may generate new perspectives in answering the question how the endocrine versus the neuronal SA derivatives develop.

6 DISCUSSION

The neural crest (NC) is a transient structure of developing vertebrate embryos that extends along the rostro-caudal axis in the dorsal part of the neural tube. The NC cells migrate and give rise to many different cell types within the organism. The derivatives of the trunk NC include chromaffin cells of adrenal glands, neurons of sympathetic ganglia and small intensely fluorescent (SIF) cells. All of them are distinct derivatives in SA cells lineage. They migrate ventrally through the rostral parts of somites and assemble first in the vicinity of dorsal aorta, then move further to destinations in sympathetic ganglia and adrenal glands. The wall of the dorsal aorta synthesizes several growth factors, among them BMP2, 4 and 7, which induce the expression of tyrosine hydroxylase (TH), a catecholaminergic marker, by the SA progenitors. The molecular mechanism underlying the development of neuroendocrine cells and neurons, distinct SA cells, is not clearly understood yet (Le Douarin and Kalcheim, 1999). For decades, it has been postulated that sympathetic neurons and adrenal chromaffin cells share a common sympathoadrenal (SA) progenitor and that glucocorticoid signalling is necessary for the specification of chromaffin cells. Analyses of glucocorticoid receptor (GR) knockout mice have challenged this concept. In the absence of GR signalling, the chromaffin cells differentiate largely normal with morphologically characteristic structures, as e.g. the typical chromaffin granules (Finotto, Krieglstein et al., 1999). Mice deficient for SF1 lack an adrenal cortex, the source of glucocorticoids, but nevertheless generate chromaffin cells (Gut, Huber et al., 2005). Thus, glucocorticoids are apparently dispensable for the differentiation of chromaffin cells.

SA lineage marker-expression analyses made by Ernsberger and colleagues have suggested that sympathoadrenal cells are heterogeneous before invading the chick adrenal glands (Ernsberger, Esposito et al., 2005). Based on these studies, it has been speculated that chromaffin cells and sympathetic neurons might originate from two different progenitor cells, which may be prespecified early in their development. We have begun to address the question whether chromaffin cells and sympathetic neurons are derived from a common sympathoadrenal progenitor at the level of neural crest or whether they develop independently from two different progenitor cells. To prove our hypothesis, we have performed several types of *in ovo* electroporation of the chick neural crest.

The distribution of chromaffin cells and sympathetic neurons within target organs does not differ after hemitube electroporation

The onset of NC cell delamination depends on somitogenesis. The emigration of neural crest cells from the NT starts at levels opposite to the epithelial somites and is organized in waves (Sela-Donenfeld and Kalcheim, 1999). Further, the delamination of neural crest cells depends on the cell cycle and the epithelial-to-mesenchymal transition. The avian NC cells emigrate from the neural tube synchronously in the S phase of the cell cycle. The G1 to S phase transition is the necessary regulatory step of delamination (Kalcheim, 2000; Burstyn-Cohen and Kalcheim, 2002). The emigration occurs in waves and we have hypothesized that sympathoadrenal cells leave the neural tube in the early steps of delamination. We further hypothesize that the progenitors of chromaffin cells and sympathetic neurons may be distinct at the level of neural tube. Therefore they may differ in their schedule of detachment from the neural tube. We hypothesize the precursors of chromaffin cells exit the neural tube before the progenitors of sympathetic neurons do.

The hemitube electroporation allowed to mark half of the neural tube and to transfect all derivatives of the neural crest. This type of electroporation of the neural tube at the different developmental stages allowed to GFP-label all neural crest derivatives remaining in the neural tube at the time point of transfection. Embryos of 15- somite to 25- somite stage were used for this type of electroporation. They were analysed at E6. The neural tube of the sympathoadrenal axial level between somite 18 to 24 was labelled. In younger embryos, the neural tube of the prospective sympathoadrenal axial level was electroporated. The delamination is known to start rostrally and to continue toward the caudal end of the embryonal axis. The first detaching cells are found opposite to the epithelial somites. When the hemitube electroporation was performed at younger stages before the delamination occurs, all premigratory sympathoadrenal progenitors were transfected within the neural tube. The electroporation of older embryos, in which the emigration of the neural crest cells has already started, resulted in labelling of neural crest cells that still remained in the neural tube. The NC progenitors that have already detached from the neural tube at the time of electroporation were not GFP-labelled. Our hypothesis suggested different timing of delamination. Assuming that the first wave was represented by the progenitors of chromaffin cells, we expected to find less GFP^+/TH^+ cells in the adrenal glands than in the sympathetic ganglia in embryos electroporated at later stages due to the fact that

the first wave of the endocrine progenitor cells was out of the NT at the time of HM electroporation.

The analysis of HM-electroporated embryos has not supported our hypothesis of two distinct progenitors. The analysis revealed an almost equal distribution of sympathetic neurons and chromaffin cells within sympathetic ganglia or adrenal glands. The sympathetic ganglia were populated by 50,58% ± 4,17 and the adrenal glands by 49,42% ± 4,16 of GFP/TH-double positive neural crest cells. No loss of GFP-labelled chromaffin cells or sympathetic neurons, respectively, was found in the embryos of different developmental stages. Our results might be influenced by the fact that sympathetic neurons are able to move two or three segments rostrally and laterally. Therefore, NC cells that originated from the axial level located more caudally or rostrally to the sympathoadrenal level might populate the area analysed in this study. It has to be taken into consideration that plasmid pCAGGS-AFP was used for HM electroporation. This plasmid is incorporated into the cytoplasm, not into the nucleus, and its concentration in the cell is diluted by every cell division. Therefore, the plasmid might be diluted to the undetectable levels when the analysis was performed. The electroporation was performed at E2 and the analysis of GFP-labelled cells at E6. . NC cells proliferate during migration, however, the proliferation rate of sympathetic neurons and chromaffin cells and their progenitors may differ. Moreover, numbers of GFP-positive cells located within the adrenal glands or sympathetic ganglia may be also influenced by cell death. However, detection of apoptosis or other forms of cell death was not performed in the context of this study.

Electroporation can be used as a method for single-cell lineage analysis

In order to directly uncover the potential of the single NC cells to form one or more derivatives, we have used several techniques of single-cell labelling. We aimed to prove the hypothesis of a common sympathoadrenal progenitor by single-cell lineage analysis of neural crest cells *in ovo*. For this purpose, different methods were performed to establish the single-cell lineage analysis. These methods were established in our laboratory in collaboration with the laboratory of Prof. Kalcheim at Hadassah University in Jerusalem: single-cell labelling was performed as (1) ventral-to-dorsal, (2) microinjection, and (3) micropipette electroporation. The highest efficiency and reproducibility of single-cell labelling was obtained by micropipette electroporation. We modified the micropipette electroporation method previously described by Haas and colleagues (Haas, Sin et al., 2001). The efficiency of single-cell transfection by micropipette electroporation reached almost 11%. Microinjection generated the lowest

efficiency (less than 1%). VD electroporation reached an efficiency of 5,3%. Eventually, micropipette electroporation was used as the method of choice for labelling of single premigratory neural crest cell.

The important factor of single-cell lineage analysis is a proof of single-cell labelling. In order to verify the number of transfected cells, immunohistochemical analysis of electroporated embryos was performed. The proportional part of single-cell labelled embryos was fixed immediately after the first control of GFP expression six hours after the transfection. The histochemical analysis revealed that the single cells had been labelled in 65% and two cell had been labelled in 35% of cases.

Results of ventral-to-dorsal electroporation

Ventral-to-dorsal electroporation was the first technique that we used in order to GFP-label a single neural crest cell in the neural tube at the “sympathoadrenal” axial level between somite pairs 18 and 24. The transfection of pCAGGS-AFP construct into a single premigratory NC cell from the first wave was performed at embryonic day two. The analysis was done at embryonic day six and the numbers of GFP/TH-positive cells in sympathetic ganglia or adrenal glands were counted. We expected to see the GFP-positive cells only in one of these two destinations in the case that distinct progenitors exist. In 5,3% of the cases of all VD-electroporated embryos, this technique resulted in the labelling of 1 - 3 cells at the dorsal aspect of the neural tube. In these cases, the labelled neural crest cells revealed the potential to form both single and multiple types of derivatives. We found the derivatives located in both destinations in sympathetic ganglia and adrenal glands, respectively, in two cases of single-cell labelled embryos. In three embryos, the GFP^+/TH^+ derivatives were found only in sympathetic ganglia. Additionally, in two other cases, only adrenal glands were populated. In two cases, GFP-positive cells were found in dorsal root ganglia only. One embryo showed three types of derivatives (cells in SG, AA and VR). Thus, this analysis did not result in a clear answer that would argue in favour or against the hypothesis of clone heterogeneity of the sympathoadrenal cell lineage. Unfortunately, the low number of experiments does not allow to test for a statistical significance. The efficiency of discrete electroporation reached about 5%. However, not every embryo that was successfully single-cell electroporated revealed GFP immunoreactivity at the time of analysis at E 6. The reason for that may be the use of the plasmid pCAGGS-AFP for transient transfection. We might miss some daughter cells of an originally GFP-labelled cell as the plasmid pCAGGS-AFP incorporates into the cytoplasm of the cells and the plasmid concentration is diluted as cells proliferate.

Several factors have been shown to play crucial roles in the labelling of discrete population of cells within the dorsal midline of the neural tube *in ovo*. One of the most critical points is placing of the electrodes. The labelling of single neural crest cell at the dorsal midline of the neural tube, where the cells are ready to emigrate, is possible by positioning of two tungsten electrodes. A very important factor is the precision of ventral-to-dorsal electroporation. The cells in the midline of the neural tube have to be marked with GFP for testing our hypothesis. Parameters influencing the efficiency of single-cell labelling are: material, shape, size and width of electrodes, the dilution of the plasmid, voltage, duration and the number of electric impulses. The majority of trials resulted in the massive transfection of the neural tube. In these trials, the transfected area extended more laterally from the midline of the neural tube; therefore not only the first wave of emigrating NC cells was labelled.

Micropipette electroporation

As the techniques of ventral-to-dorsal electroporation or microinjection provided only low efficiency, we decided to perform the single-cell labelling by micropipette electroporation. Single-cell lineage labelling resulted in dual progeny located in both sympathoadrenal destinations in most of the cases. It is important to note that the GFP-labelled cells were found in the location of adrenal gland at the level of electroporation whereas the derivatives located in the sympathetic ganglia were found rostrally to the level of electroporation (see above). In addition to the dual progeny found in the majority of analysed embryos, the single-cell lineage analysis resulted in single cell type derivatives in three analysed embryos. Only one embryo that was electroporated opposite to the 23rd somite level, which corresponds to the caudal end of sympathoadrenal level, showed the derivatives in adrenal gland only. Two embryos, that were electroporated opposite to the 19th somite level, formed derivatives located only in sympathetic ganglia. In more than 87%, the results demonstrate that a single neural crest cell gives rise to both types of derivatives located in the adrenal medulla and sympathetic ganglia, respectively. These findings strongly support the hypothesis of a common sympathoadrenal progenitor cell. The neural crest cells of the sympathoadrenal lineage have potential to form different types of catecholaminergic cell types. However, it remains still unknown how the different adrenergic phenotypes arise from neural crest. It is unclear what factors are involved in chromaffin cell fate determination.

The main interest was focused on the sympathoadrenal single-cell lineage analysis. Over the above-mentioned results, the single-cell lineage analysis has provided findings regarding the development of other neural crest cell types. After the single-cell labelling, we have analysed embryos, which formed derivatives located just in dorsal root ganglia or just in ventral root. The embryos with derivatives placed in both locations, the dorsal root ganglia and the ventral root, were found as well. The single-cell analysis of the neural crest cells suggests a limited potential of premigratory neural crest cells located in the neural tube, to form multiple cell types.

To overcome the problem of plasmid-concentration dilution upon the cell division, we have used the plasmids–construct that is stably expressed in cells for micropipette electroporation. This construct is composed of two plasmids pT2K-CAGGS-EGFP, which encodes an EGFP cassette flanked by transposone-Tol2, and pCAGGS-T2TP expressing transposase-Tol2. Upon co-electroporation, the Tol2-flanked EGFP cassette is cut and transposed into the genome of the host cell by transposase activity (Sato, Kasai et al., 2007). The EGFP expression is thereby not decreased as the cells proliferate. Nevertheless, not every embryo that has shown the EGFP expression in a single cell at E2-E2,5 maintained the GFP-expression till E6. A possible explanation for this fact is that the labelled cells might undergo apoptosis or other forms of cell death before the final analysis was performed.

Sympathetic ganglia contain a portion of chromaffin-like cells

Recently, it has been found that 25% of the avian sympathetic ganglion cells express catecholaminergic but not neural markers. These cells have been called chromaffin-like cells (Huber K. *et al*., 2008). We have shown that trunk sympathetic ganglia of chicken embryos contain about 23% of chromaffin-like cells. Our results obtained from the analysis of E6 chicken sympathetic ganglia are in accordance with the previously described findings in E8 chicken embryos.

Single neural crest cell give rise to both the sympathetic neurons in sympathetic ganglia and the chromaffin cells of adrenal gland

Our finding of the dual progeny after single-cell micropipette electroporation in most of the cases strongly supports the hypothesis of a common sympathoadrenal cell lineage progenitor meaning that the fate of chromaffin cells and sympathetic neurons,

respectively, is not predetermined at the level of the neural crest. We have used tyrosine hydroxylase as a marker for catecholaminergic cells. Therefore we were not able to differentiate between sympathetic neurons and chromaffin-like cells in the sympathetic ganglia. To distinguish these two populations, we used neurofilament-M as the specific marker for neurons. In seven single-cell-labelled embryos the combined immunofluorescence (TH/GFP) and *in situ* hybridisation analysis (NF-M) was performed. The results revealed that a single-cell gave rise to both types of derivatives. The TH/ GFP-labelled derivatives located in sympathetic ganglia were identified as NF-M-positive sympathetic neurons whereas the TH/ GFP-positive cells of adrenal gland as chromaffin cells were NF-M-negative. We therefore tentatively conclude that the dual progeny of crest cells found in adrenal gland and sympathetic ganglia truly represents chromaffin cells and neurons, respectively.

Notch signalling

Sympathoadrenal progenitors generate diverse types, including endocrine cells, neurons, and an intermediate cell type, the SIF cell. The cues determining the fate of chromaffin cell is still unclear. Notch/Delta signalling has been shown to play a role in cell-to-cell communication. Recently, the development of early sympathetic and sensory ganglia has been shown to depend on Notch signalling (Tsarovina, Schellenberger et al., 2008). HES1, a gene that is actively regulated by Notch1, inhibits the function of Mash1 (Sasai, Kageyama et al., 1992). Delta-like1 (Dll1) is a direct target gene of Mash1, the early sympathoadrenergic marker. The expression of Mash1 and Dll1 is upregulated in Insm1 knockout mice but no upregulation was observed in the expression levels of Notch target gene such as Hes1, Hes5 (Wildner, Gierl et al., 2008). Therefore, we have examined the expression pattern of Notch/Delta signalling members in the early developing adrenal glands and sympathetic ganglia. The expression of Delta-like 4 has been studied previously. The analysis has found the Dll4-mRNA in the arterial type of endothelial cells and also in paraaortic bodies during chicken development (Nimmagadda, Geetha-Loganathan et al., 2007). Our analysis showed a similar expression pattern, however, the signals showed lower intensity than was previously shown. The expression of Dll1-mRNA at E3 was located in the area between the dorsal aorta and nephric (mesonephric) duct, where the adrenal anlagen form in chicken. The Dll1 expression did not persist in this area till E6. The expression of Hairy1-mRNA was located along the migration route of neural crest cells, at the prospective area where the dorsal root ganglia form at E4. At later stages, at E6, the expression of both Hairy1 and Hairy2 was obvious in the area of adrenal anlagen and

primary sympathetic chain. However, an analysis with additional markers still needs to be done in order to identify the type of cells where the expression of Notch signalling members is located. The marker of neural crest cells HNK1 and the marker of catecholaminergic cells TH would provide initial information about the cell type. A further approach that would examine the possible role of Notch signalling in the development of sympathoadrenal lineage represents the manipulation of Notch-signalling function. We suggest to modulate the function of Notch by electroporating the Notch-repressing or -inducing constructs into the neural crest cells and to monitor the development of sympathetic neurons and adrenomedullary chromaffin cells.

Vascular pattern of adrenal gland and sympathetic ganglia

It is assumed that sympathoadrenal cells differentiate into sympathetic neurons and adrenal chromaffin cells upon the influence of environmental factors, which are derived from their final destinations or are present on the migration route. Upon single-cell labelling at the level of the neural tube, we have found that both sympathetic neurons and chromaffin cells are derived from the same sympathoadrenal precursor in the majority of the cases. Nevertheless, the factors triggering the fate of chromaffin cell differentiation are still unknown. Based on the fact that sympathoadrenal cells are a heterogeneous population of cells before the invasion of their final destination, the fate determination must happen between delamination and settling of the vicinity of dorsal aorta. It has been found that endocrine chromaffin and SIF cells are located in the close proximity to the blood vessels. We therefore studied the vascular pattern in the developing avian embryos. Further, we were interested whether HNK-1-positive neural crest cells reach the presumptive area of adrenal gland and primary sympathetic ganglia before the vascular pattern develops in these locations. We have found that HNK1-positive neural crest cells migrate to the dorsal aorta already at E2-E2,5 in the quail model. The endothelial cells were present in the prospective area of sympathetic ganglia and adrenal glands at E3 – E3,5. The vascular pattern of both areas populated by catecholamine-producing cells qualitatively differs as early as at E4-E4,5. The endothelial cells of adrenal anlagen are placed within the developing organ whereas the endothelial cells of the sympathetic primordium are preferentially located at the margins of the developing ganglia. These preliminary results require further investigations of the molecular difference in vasculature of sympathetic ganglia and adrenal gland. Recently, a vascular growth factor specific for endocrine gland endothelial cells, EG-VEGF, was found. Vascular endothelial growth factor receptor 3 (VEGFR-3) was detected in the endothelium of lymphatic vessels and in the

fenestrated capillaries of several organs including kidney glomeruli and endocrine glands (Partanen T. et al., 2000). High levels of VEGF-C, a ligand of (VEGFR-3 and (VEGFR-2, are expressed in neuroendocrine cells that are lined by fenestrated capillaries. That could play a role in the interaction of neuroendocrine cells and fenestrated endothelium (Partanen T. et al., 2000). The factors that may be differently expressed by endocrine or ganglionic endothelium, respectively, could influence the fate of sympathoadrenal cells.

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8 LIST OF ABBREVIATIONS

µ	micro
AA	adrenal anlagen
Ach	acetylcholine
AEC	3-amino-9-ethylcarbazol
AG	adrenal gland
AMP	ampicilin
Ascl1	the vertebrate homolog of Drosofila achaete scute complex
BMP	bone morphogenetic protein
bp	basepair
cDNA	complementary DNA
CNS	central nervous system
DA	dopamine
DNA	deoxyribonucleic acid
DRG	dorsal root ganglia
E	epinephrine (adrenaline)
E2	embryonic day 2
E. coli	Escherichia coli
EtOH	ethanol
DA	dorsal aorta
DAPI	4',6-diamidino-2-phenylindole
DBH	dopamine β-hydroxylase
dHand (Hand1/2)	heart and neural crest derivatives expressed 1/2
DIG	digoxigenin
Gata2/3	GATA binding protein 2/3
GFP	green fluorescent protein
GR	glucocorticoid receptor
HNK1	human natural killer antigen
IHC	immunohistochemistry
ISH	*in situ* hybridisation
kb	kilobase
l	litre
LB	Luria Bertani
m	mili
min	minute
mRNA	messenger RNA

NC	neural crest
NE	norepinephrine (noradrenaline)
NGF	nerve growth factor
NT	neural tube
No	notochord
O/N	over night
pBS	plasmid Bluescript
Phox2a/b	Paired-like (aristaless) homeobox 2a/b
PNMT	phenylethanolamine-N-methyltransferase
PNS	peripheral nervous system
RNA	ribonucleic acid
RT	room temperature
s	second
SA	sympathoadrenal
SG	sympathetic ganglion
SIF cell	small intensely fluorescent cell
TH	tyrosine hydroxylase
VR	ventral root

9 LIST OF FIGURES, TABLES AND GRAPHS

FIGURES

TABLES

GRAPHS

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